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By

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DEDICATION

"For from him and through him and to him are all things. To him be the glory forever! Amen." Romans 11:36

Lisa,
Thank you for enduring. I know it has not been what we originally planned.

Madisen,
For all the weekends you spent in lab with me, thank you for being such a patient, sweet and loving daughter.

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ABSTRACT

THE REGULATION OF RNA POLYMERASE I-MEDIATED TRANSCRIPTION IN FOREBRAIN NEURONS

Scott Carl Smith

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Ribosomal biogenesis failure may contribute to neurodegenerative diseases, while its excessive activation has been shown to drive tumor growth. As ribosomal production is initiated and regulated by the Pol1-mediated transcription of rRNA genes in the nucleolus, the latter process had the attention of most researchers interested in the dysregulation of ribosomal biogenesis in disease. When this work began, regulation of Pol1-mediated transcription in neurons had been poorly characterized. The goal of this research has been to better define factors and signaling pathways that regulate neuronal Pol1 activity. The first hypothesis tested was that DNA damage induced by a DNA topoisomerase poison, etoposide, blocks neuronal Pol1. Intracarotid delivery of etoposide to adult rats resulted in Pol1 inhibition in the neocortex; however no apoptosis was found. In neonate rats that received intracerebroventricular injections of etoposide we observed inhibition of Pol1 in neurons in the neocortex and hippocampus. Neuronal apoptosis was observed in these brain structures following etoposide treatment. Therefore, these results confirm that neuronal Pol1 is sensitive to etoposide-induced DNA damage and that such a sensitivity is present in both young and mature neurons of whole rats. These results also demonstrate that Pol1 inhibition is distinct from the

developmentally-restricted apoptotic response to etoposide. While working on these studies, we made the surprising finding that doses of etoposide induced Pol1-mediated transcription in cultured cortical neurons and whole rat neonate brains. Thus, low doses of etoposide that were 3 orders of magnitude lower than those inducing Pol1 block apoptosis and were sufficient to activate Pol1 and the DNA double strand break response, including autophosphorylation of the DNA damage signaling kinase Ataxia-telangiectasia Mutated (ATM). Therefore, it was hypothesized that ATM activated neuronal Pol1. Indeed, pharmacological inhibition of ATM with KU55933 blocked Pol1 activation in response to low concentrations of etoposide or to another topoisomerase-II inhibitor, ICRF-193. Basal levels of nucleolar transcription were also suppressed. Moreover, Pol1-driven transcription was reduced in the cerebellum of 5-week old ATM-null mice. Additionally, pharmacological inhibition of ATM reduced neuronal Pol1 activation in response to the neurotrophin, BDNF, or to increased synaptic activity. Consistent with the notion that BDNF-mediated neurite outgrowth requires Pol1, KU55933 appeared to reduce BDNF-mediated neurite outgrowth. Similar effects on ribosomal biogenesis and neuronal growth were observed with a pharmacological inhibitor of the ATM-related kinase mTOR. Finally, ATM inhibition reduced Pol1 activation in serum-stimulated human cell lines. These findings identified ATM as a novel regulator of Pol1. They further confirmed that in neurons, as in non-neuronal cells, mTOR also regulates Pol1. My results suggest that at least some consequences of ATM deficiency in humans, including neurodegeneration or impaired proliferation, result from insufficient ribosomal biogenesis.

Taken together, these studies filled important gaps in the knowledge of Pol1 regulation in neurons by demonstrating nucleolar transcription sensitivity to DNA damage in whole animal brains and by defining signaling pathways contributing to its activation by neurotrophic stimulation. My findings suggest that DNA damage or loss of TM may contribute to neurodegeneration by decreasing ribosomal biogenesis.

TABLE OF CONTENTS

TABLE OF FIGURES

CHAPTER I

INTRODUCTION

First described in neurons in 1839 by a pupil of Jan Evangelista Purkinje, Gabriel Valentin (Harris, 2000), the primary function of the nucleolus was only definitively agreed upon in 1965 (Pederson and Tsai, 2009). Within the nucleus, Valentin described a "sharply rounded object which thus constitutes a kind of nucleus within a nucleus." Indeed, the definition of the word, "nucleolus" is nucleus within a nucleus. Nucleolar function, identified primarily in transformed cells, was the place of ribosomal biogenesis (Pederson and Tsai, 2009). Though key findings in the mid 1960's led many researchers to agree upon nucleolar function, it took a meeting in Uraguay by groups of scientists who had watched the development of nucleolar function to officially claim ribosomal biogenesis to the location of the nucleolus (Pederson and Tsai, 2009). In recent years with the development of advanced proteomics tools it was identified that only approximately 30% of the more than 4500 proteins residing in the nucleolus are directly associated with ribosomal biogenesis. This has been interpreted to mean that though ribosomal biogenesis is a function of the nucleolus, it is not the only function. The plurifunctional nucleolus is regularly revealing new and exciting functions to the scientists who study it nearly 200 years after its description in animal cells was first published.

Despite being the first described animal cell with a nucleolus, the nucleolus of the postmitotic neuron has gained attention only recently. The cellular function of neuronal nucleoli, the regulation of Pol1 transcription and the responsiveness to stress are all

present in neurons as well as cycling cells; yet the fates of neurons are different from cycling cells. Only a small percentage of the total neuronal population of the brain regenerate, thus the longevity of neurons is of the utmost importance in healthy development and aging. Further, in neurons actively translating ribosomes are present in the soma, axons and dendrites of neurons. The functions of proteins being synthesized locally include axon guidance (Campbell and Holt, 2001), synapse formation (Schacher and Wu, 2002), neuronal survival (Howe and Mobley, 2005; Cox et al., 2008), and synaptic plasticity (Ostroff et al., 2002). Indeed, bidirectional transport of locally translated proteins is a means of signaling the great distance between the soma and synapse (Howe and Mobley, 2005; Cox et al., 2008; Lin and Holt, 2008). In addition, morphogenesis of neurites requires transcription leading to ribosomal biogenesis (Gomes et al., 2011). Therefore the ongoing synthesis of ribosomes, the half-life of which is 7-15 days (Stoykova et al., 1983), is of utmost importance in neurons. The generation of new ribosomes may be important in maintaining neuronal function as the accumulation of oxidative damage has been identified in RNA of ribosomes of Alzheimer's disease patients (Honda et al., 2005; Ding et al., 2006). Most steps of ribosome synthesis occur in the nucleolus, a transcription-dependent nuclear structure.

Nucleolar transcription of rRNA genes initiates ribosomal biogenesis and is tightly regulated to adjust the rate of ribosomal production to cellular needs. Pathways that signal cellular growth, such as mammalian target of rapamycin (mTOR), mitogen-activated protein kinases (MAPK), extracellular signal-related kinase (ERK) and p90 ribosomal S6 kinase (RSK), and cyclin-dependent kinases (CDKs) directly regulate the nucleolus (Grummt, 2003; Rubbi and Milner, 2003; Mayer et al., 2005; Mayer and

Grummt, 2005; Boulon et al., 2010; Drygin et al., 2010; Hetman et al., 2010). Nucleolar transcription is also affected by cellular stress. Nucleolar transcription is inhibited by DNA damage, reactive oxygen species or nutrient starvation (Rubbi and Milner, 2003; Mayer et al., 2005; Mayer and Grummt, 2005; Kruhlak et al., 2007; Kalita et al., 2008). The nucleolar sensitivity to stress provides a convenient stress signaling mechanism. Stress-induced inhibition of ribosomal biogenesis preserves cellular energy (Grummt and Voit, 2010) and disrupts nucleolar integrity thereby allowing the passive diffusion of nucleolar-residing proteins that can mediate various stress responses throughout a cell, such as stabilization of p53 (Rubbi and Milner, 2003; Andersen et al., 2005; Mayer and Grummt, 2005). However, long-term reduction of ribosomal biogenesis may have permanent negative consequences on the cell. The pathology of ribosomopathies, whose origins are in decreased ribosomal biogenesis, provides evidence for this notion (Lipton and Ellis, 2009; Burwick et al., 2011). In neurons, nucleolar transcription is necessary for neuronal development and maintenance (Parlato et al., 2008).

The present studies began as an attempt to identify DNA damaging agents that affected Pol1 transcriptional activity in neurons. With such tools, we planned to test whether DNA damage-induced nucleolar disruption occurs in both developing and mature brain. While conducting these studies we made a surprising discovery that a DNA damage signaling protein kinase, Ataxia-telangiectasia mutated (ATM) stimulates nucleolar transcription. Therefore, we discovered a novel regulator of nucleolar transcription that functions independently of canonical Pol1-mediated regulatory signaling pathways.

The nucleolus and transcription of ribosomal RNA genes

The nucleus is an organelle that encompasses non-membrane-bound structures, such as Cajal bodies, PML bodies, and others, all of which represent functional compartmentalization (Emmott and Hiscox, 2009). Biogenesis of the nucleolus is transcription dependent (Rubbi and Milner, 2003). The nucleolus contains a vast proteome as well as nucleic acids with a particular enrichment in RNA (Boisvert et al., 2007). Though once believed to be only a constitutive ribosome producer, the nucleolus is now known to be a highly dynamic compartment. It is responsive to growth stimuli (Grummt, 2003) and stress signals (Mayer et al., 2005), and is involved in such diverse functions as DNA repair (Boisvert et al., 2007), stress sensing (Mayer and Grummt, 2005), RNA splicing and processing (Tschochner and Hurt, 2003; Andersen et al., 2005; Wahl et al., 2009).

RNA Polymerase-1 (Pol1) is the nucleolar RNA polymerase which is the only mediator of nucleolar transcription of the repeated nucleolar genes encoding 18S, 28S and 5.8S rRNAs (ribosomal DNA, rDNA). Each of several hundred nucleolar rDNA units can be transcribed into a 45S precursor-rRNA (pre-rRNA), which is rapidly processed to the 18S, 28S, and 5.8S transcripts (Fig.1). Human rRNA genes are organized in tandem repeats at nucleolar organizer regions (NORs) on the p12 band of the acrocentric chromosomes (13, 14, 15, 21, and 22) (Grummt, 2003; Mais et al., 2005; Drygin et al., 2010). Ribosomal biogenesis begins with the transcription of rDNA by Pol1 at NORs in the fibrillar center (FC) of the nucleolus. The processing of pre-rRNA is accomplished by small nucleolar ribonucleoproteins (snoRNPs) in the dense fibrillar component (DFC) of the nucleolus, just exterior to the FC (Boisvert et al., 2007). The

final maturation of ribosomal transcripts and assembly with ribosomal proteins occurs in the granular component (GC) of the nucleolus, the region furthest from the FC and closest to the nucleoplasm. The 5S rRNA transcript, transcribed by RNA Polymerase-III outside the nucleolus, assembles with the 28S and 5.8S rRNA transcripts and ribosomal proteins to form the 60S subunit (Boisvert et al., 2007; Strunk et al., 2011). The mature 18S rRNA transcript assembles with ribosomal proteins to form the 40S subunit. The 40S and 60S subunits are individually exported to the nucleoplasm and subsequently assembled together to form the mature ribosome (Boisvert et al., 2007).

Ribosomal biogenesis is extremely costly to the cell (Warner, 1999). In *S.cerevisiae* approximately 2000 ribosomes are generated per minute. The toll this takes on the cell accounts for 60% of the total cellular transcriptional output, and is the sum of no less than 50% of RNA Polymerase II-mediated transcription. PolII is required to transcribe 137 mRNAs, accounting for 78 ribosomal proteins that are directly involved in ribosomal biogenesis. Indeed, enzymes such as kinases that regulate ribosomal biogenesis are not even accounted for in these calculations (Warner, 1999).

Synthesis of rRNA is highly regulated as would be expected for such an energy-requiring process. Ribosomal biogenesis requires the cooperation and activation of multiple co-factors (Fig.2). Pre-initiation complex assembly requires the recruitment of upstream binding factor (UBF) and transcription initiation factor – IB (rodent)/promoter selectivity factor (humans) (TIF-IB/SL1) (Drygin et al., 2010). DNA binding by the UBF high mobility group (HMG) box allows the bending of approximately 140 base pairs (bp) which creates an enhancosome structure to promote transcription (Bazett-Jones et al., 1994). The UBF-mediated enhancosome structure stabilizes TF-IB/SL1 promoter binding

and recruits Pol1 (Drygin et al., 2010). UBF also engages in the promotion of transcriptional elongation. The assembly of a productive transcription initiation complex depends on Pol1 recruitment. TIF-IB, made up of the TATA binding protein and TATA binding protein-associated factors, TAF_I proteins, recruits Pol1. The interaction between TAF_Is of TIF-IB/SL1 and TIF-1A creates a bridge between TIF-1B/SL1 and RPA43, a subunit of Pol1 (Grummt, 2003; Drygin et al., 2010).

Epigenetic modification of rDNA can also enhance or repress nucleolar transcription. Though not as rapid as the transcriptional regulation of active rDNA by signaling pathways, epigenetic modification can regulate the transcriptional output of the nucleolus by recruiting silenced rDNA to an active state or vice versa (Grummt and Ladurner, 2008). Importantly, epigenetic modification of rDNA can be mediated by cell-extrinsic factors that are critical to cell growth, such as glucose levels (Murayama et al., 2008). Promoters of silenced rRNA genes are hypermethylated at CpG residues. The nucleolar remodeling complex (NoRC) silences rDNA by recruiting DNA methyltransferase and histone deacetylase activity to the promoter (McStay and Grummt, 2008).

Figure 1

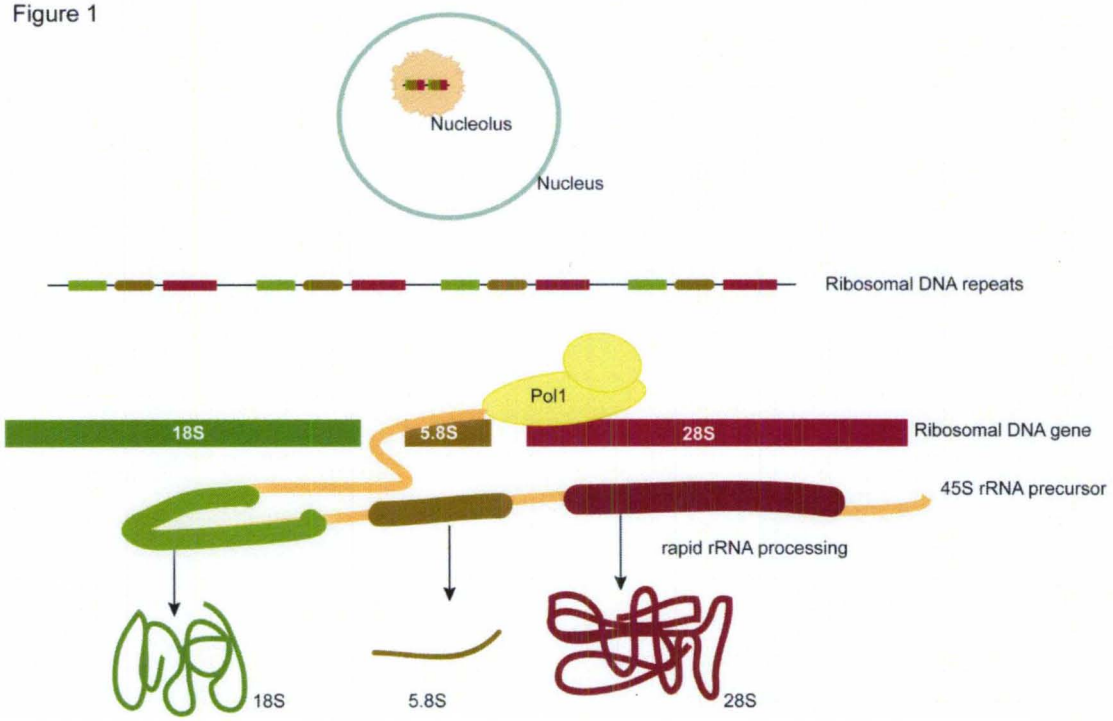

Figure 1: The nucleolus is a transcription-mediated organelle.
Ribosomal DNA (rDNA) is organized in tandem repeats in nucleolus. Transcription of rDNA is conducted by RNA Polymerase 1, which produces the 45S precursor rRNA (pre-rRNA or 45S rRNA). Rapid processing of rRNA results in the mature 18S, 5.8S and 28S transcripts.

Figure 2

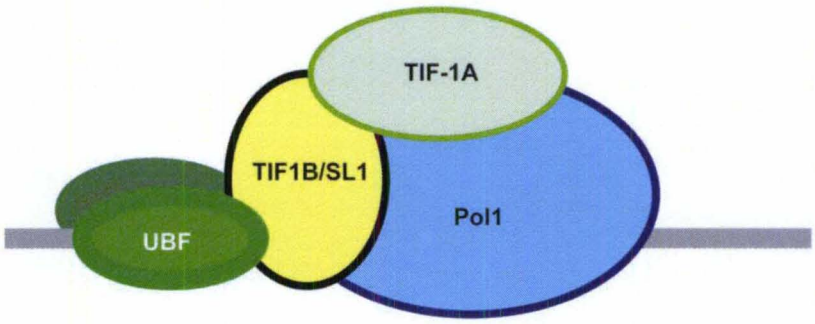

Figure 2. A diagram of the transcription initiation unit as described in the text.
A productive transcription initiation complex requires the UBF- TIF-1B/SL1 stabilization on the promoter, leading to the recruitment of Pol1. Interaction between TIF-1B and Pol1 is mediated by TIF-1A.

Role of the nucleolus in cell growth

During neuronal development, establishing connections of individual neurons to one another is dependent upon extracellular signals, such as growth factors, electrical activity and neurotransmitters. Local protein synthesis is often required to respond to many such signals (Alvarez et al., 2000; Ethell and Pasquale, 2005; Howe and Mobley, 2005; Matus, 2005; Lin and Holt, 2008; Gumy et al., 2010; Jan and Jan, 2010; Mikl et al., 2010; Neal et al., 2010; Gutierrez and Davies, 2011). The response to these signals results in the activation of intracellular signaling cascades that stimulate neurite outgrowth and synaptogenesis (Miller and Kaplan, 2003). Thus, as an initiator of ribosomal biogenesis, nucleolar transcription is critical for cellular growth. An important mediator for such regulation is TIF-1A with a variety of signaling pathways that converge on it (Grummt, 2003). mTOR regulation of Pol1-mediated transcription is accomplished through

phosphorylation of TIF-1A. mTOR promotes the activation of Pol1-mediated transcription by phosphorylating S44 and inhibits Pol1 transcription by phosphorylating S199 (Mayer et al., 2004). ERK1/2 regulation of TIF-1A potentiation occurs with the cooperation of p90 ribosomal S6 kinase (RSK). Mitogen stimulation occurs through receptor tyrosine kinase (RTK) and the signaling cascade follows through RAS, RAF, MEK and finally to ERK1/2. ERK1/2 then activates RSK, which phosphorylates TIF-1A at S649. That is followed by ERK1/2-mediated phosphorylation of TIF-1A at S633. Both phosphorylations are required for ERK1/2-mediated TIF-1A activity.

Brain-derived neurotrophic factor (BDNF) is a major neurite outgrowth-promoting neurotrophin for forebrain neurons. BDNF binds TrkB receptors that signal to TIF-1A via RAS and ERK1/2 pathways to regulate neurite outgrowth and induce primary dendrite formation through the activation of MAP kinase and PI3K signaling pathways (Dijkhuizen and Ghosh, 2005). BDNF-mediated outgrowth of neurites has recently been shown to be TIF-1A-dependent via ERK1/2 signaling (Gomes et al., 2011). In that study, the constitutive activation of MKK1 induced neurite outgrowth similar to BDNF-induced outgrowth in a TIF-1A-dependent manner. Similarly, constitutively active TIF-1A also induced neurite outgrowth. Indeed, such findings suggest that Pol1 is a critical pro-growth target of ERK1/2 (Gomes et al., 2011).

mTOR signaling that is activated by insulin and neurotrophins through RAS-dependent activation of Phosphatidylinositol 3-kinase (PI3K) also contributes to neurite outgrowth and branching. While its role in promoting neurite outgrowth has been attributed to its effect on translation (Jaworski et al., 2005; Swiech et al., 2008), mTOR is also a major regulator of Pol1 and ribosomal biogenesis by targeting TIF1A (Grummt,

2003; Drygin et al., 2010). However, whether that Pol1-driven activity of mTOR is essential for neurite outgrowth remains to be determined.

In addition to TIF1A, other initiation complex co-factors are regulated by growth promoting signaling pathways. UBF phosphorylation by ERK1/2 in response to mitogenic signals at T117 and T201 robustly modifies the DNA architecture of the enhancosome to promote Pol1 transcription through it (Stefanovsky et al., 2001; Stefanovsky et al., 2006). mTOR phosphorylates UBF at its C-terminus thereby promoting the interaction between UBF and TIF-IB/SL1 in response to growth signals and nutrients (Hannan et al., 2003). In proliferating cells, cyclins and their kinases activate UBF during cell cycle progression. Cyclin E/Cdk2 and Cyclin A/Cdk1 phosphorylate UBF during interphase on S484 by Cyclin D/Cdk4 and Cyclin E/Cdk2 and on S388 (Drygin et al., 2010 86).

Nucleolar roles beyond ribosomal biogenesis

A vast proteome in the nucleolus has been identified suggesting that the functional significance of the nucleolus goes beyond ribosomal biogenesis (Andersen et al., 2005). Annotations of nucleolar proteins have implicated the nucleolus in processes such as apoptosis, cell stress signaling, DNA repair, signal recognition particle biosynthesis, telomere function, aging, and synaptic activity, among others (Pederson, 1998; Mayer and Grummt, 2005; Jordan et al., 2007). Proteins directly related to ribosomal biogenesis account for only 30% of the nucleolar proteome (Andersen et al., 2005; Boulon et al., 2010). Proteins can freely diffuse in and out of the nucleolus since there is no membrane boundary, therefore the retention of many proteins to the nucleolus

depends on the affinity to bind relatively immobile components of that structure (Pederson and Tsai, 2009). Conversely, upon inhibition of Pol1, many nucleolar proteins diffuse throughout the nucleoplasm and/or the cytosol. One such protein, Nucleophosmin/B23, is a multi-functional histone chaperone protein (den Besten et al., 2005; Maggi et al., 2008; Nalabothula et al., 2010; Colombo et al., 2011). It is required for the correct assembly and disassembly of nucleosomes (Maggi et al., 2008; Colombo et al., 2011), participates in shuttling pre-ribosome particles from their point of synthesis to the cytoplasm (den Besten et al., 2005), and after its nucleolar release following Pol1 inhibition, is involved in cell cycle arrest (Nalabothula et al., 2010) and $p19^{ARF}$ stabilization (Colombo et al., 2011).

The nucleolus as a stress sensor

rDNA transcription is highly sensitive to stress including exogenous DNA damage, oxidants, hypoxia, heat shock (Rubbi and Milner, 2003), or nutrient starvation (Mayer et al., 2004). The mechanisms associated with the suppression of rDNA transcription have been investigated only recently (Boulon et al., 2010). Following nutrient starvation, increasing AMP/ATP ratio activates a protein kinase AMPK, which carries out inhibitory phosphorylation of TIF1A at S635 (Grummt, 2003; Hoppe et al., 2009). In response to exogenous oxidative stress, nucleolar transcription suppression is dependent upon c-jun N-terminal protein kinase 2 (JNK2) (Mayer et al., 2005) that also targets TIF1A. Similarly, Pol1-mediated transcriptional inhibition following irradiation or etoposide has been shown to be ATM-dependent (Kruhlak et al., 2007).

As the nucleolus is a transcription-dependent structure, inhibition of Pol1 disrupts nucleolar integrity (Olson, 2004; Boulon et al., 2010). The disrupted nucleolar integrity

has been characterized ultrastructurally as the separation of the FC and GC. In addition, many nucleolar proteins are released from the nucleolus critically contributing to the stress responses such as p53-mediated apoptosis (Boulon et al., 2010). Under basal conditions p53 is continuously ubiquitinated by the E3 ligase, MDM2, and thereafter degraded in the cytoplasm. Upon nucleolar disruption, ARF is released to the nucleoplasm where it binds up MDM2 thereby preventing its association with p53. This allows p53 to accumulate in the nucleus and be a responsive genotoxic effector (Rubbi and Milner, 2003). Nucleophosmin also binds ARF thereby preventing its association with E3 ligases (Colombo et al., 2011). In addition, ribosomal proteins L5, L11, L23 and S7 have been shown to directly associate with MDM2 to prevent p53 degradation. Such interactions are promoted by nucleolar disruption (Boulon et al., 2010). Alternatively, increased translation of L11 can promote p53 accumulation even if nucleolar structure is undisturbed. L11 mRNA contains a 5'-terminal oligopyrimidine sequence that allows translation of a subset of mRNAs under global translation inhibition. Indeed, 40S ribosomal subunit biogenesis inhibition drove L11 mRNA translation leading to p53 acetylation in the absence of nucleolar disruption (Fumagalli et al., 2009; Boulon et al., 2010). Post-translational modifications of p53 can promote its accumulation following stress. For instance, acetylation of p53 and MDM2 by p300/CBP prevents their binding following nucleolar stress. Under non-stress conditions, however, MDM2 suppresses p53 acetylation (Boulon et al., 2010).

Neurodegenerative disease and impaired nucleolar transcription

Chronically impaired nucleolar transcription has negative consequences on non-proliferating cells (Kalita et al., 2008; Parlato et al., 2008; Hetman et al., 2010; Rieker et al., 2011). Impaired nucleolar transcription by the genotoxin, camptothecin, or the small hairpin RNA knockdown of the nucleolar transcription co-factor, *Tif-1a*, causes neuronal nucleolar disruption and apoptotic cell death in cultured cortical neurons from newborn rats (Kalita et al., 2008). Similarly, ablation of *Tif-1a* in the developing CNS causes massive apoptosis of neuronal precursors that prevents brain development (Parlato et al., 2008). In both dissociated neurons and in the whole animal, the accumulation of p53 in neurons is a trigger for relatively rapid apoptotic death upon Pol1 inhibition. Conversely, if *Tif-1a* is eliminated from the mature nervous system, slow-progressing neurodegeneration occurs. The first evidence becomes noticeable one month following the induced knock out of the *Tif-1a* gene and continues for at least 30 weeks, at which time 80% of neurons are lost (Parlato et al., 2008). This is consistent with the long half-life of neuronal ribosomes (Stoykova et al., 1983). Impaired nucleolar transcription likely hinders ribosome production and renewal leading to ribosomal failure, insufficient protein synthesis and subsequent neuronal atrophy (Hetman & Pietrzak, 2011, in press). In addition, the disruption of nucleolar integrity may allow the diffusion of nucleolar proteins into the nucleoplasm that have chronic negative effects on the cell, such as the accumulation of p53 (Emmott and Hiscox, 2009) (Fig. 3).

Evidence of accumulated oxidative stress is prevalent in many neurodegenerative diseases (Zhang et al., 1999; Sayre et al., 2001; Ding et al., 2005; Honda et al., 2005; Barber et al., 2006; Ding et al., 2006; Markesbery and Lovell, 2006; Brooks, 2007; Fishel

et al., 2007; Lovell and Markesbery, 2007b; Martin, 2008b; Nunomura et al., 2009; Kirshner et al., 2011). Indeed, in PD and AD, there is evidence that RNA oxidation occurs in the cytoplasm and affects ribosomes (Zhang et al., 1999; Ding et al., 2005; Honda et al., 2005; Ding et al., 2006; Nunomura et al., 2009). In AD, this has caused ribosomes to have reduced protein translation efficiency thereby preventing sufficient cellular maintenance and contributing to the vicious cycle of AD pathology (Ding et al., 2005). Conversely, increased susceptibility to oxidative stress may stem from impaired nucleolar transcription (Rieker et al., 2011).

The ablation of *Tif-1a* specifically in developing dopaminergic neurons also causes a substantial reduction in tyrosine hydroxylase-positive neurons from the substantia nigra and striatum only 15 days after birth. At postnatal day 30 there were significant locomotor deficits associated with ablation of *Tif-1a* in dopaminergic neurons (Rieker et al., 2011). As may be expected, ablation of *Tif-1a* in mature dopaminergic neurons using a tamoxifen-inducible Cre-recombinase caused a reduction in dopaminergic neurons as early as seven weeks following initial tamoxifen injections, reduced dopamine content of dopaminergic neurons, and within 12 weeks produced locomotor deficits (Rieker et al., 2011). These results point to the necessity of Pol1-mediated transcription for dopaminergic neuronal maintenance, suggesting a possible relationship between nucleolar insufficiency and PD. In fact, disrupted nucleoli in dopaminergic nigral neurons have been documented in postmortem brain samples from PD patients (Rieker et al., 2011).

Excessive epigenetic silencing of rDNA has also been linked to the hippocampal atrophy in severe depression of developmental origin and AD. Hypermethylation of

rDNA promoter CpGs was observed in the postmortem hippocampus of suicide victims with depression and a history of childhood abuse (McGowan et al., 2008). Similar hypermethylation has been documented in the cerebral cortex of AD patients and in the elderly with mild cognitive impairment, which often represents early stage AD (Pietrzak et al., 2011a). These findings may suggest active rDNA silencing mediated by cellular stress over many years.

Figure 3

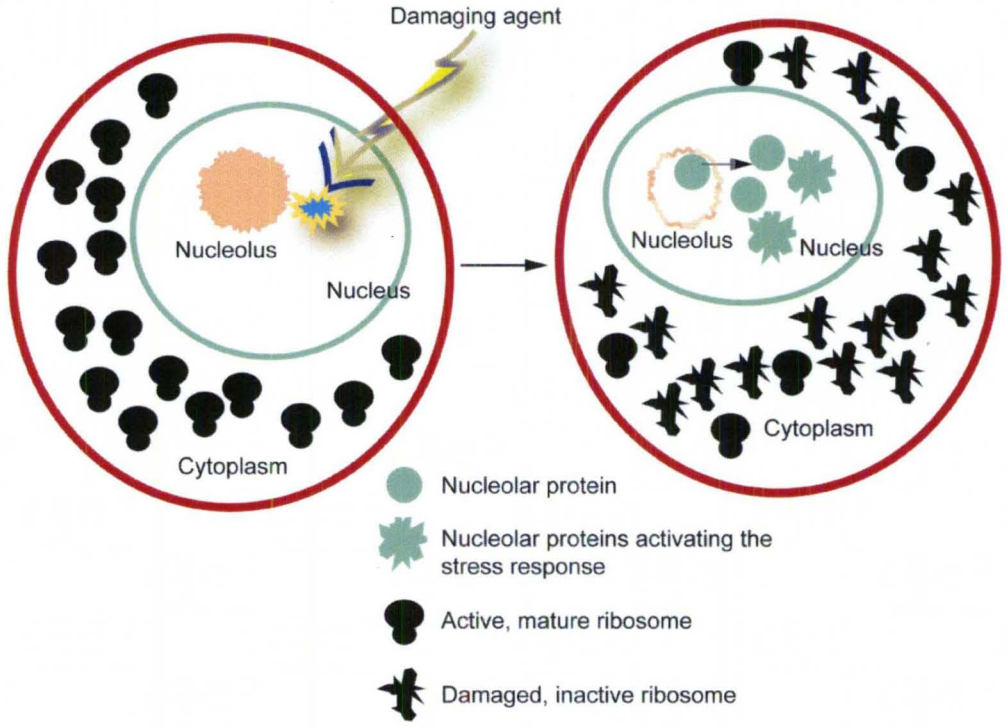

Figure 3: Potential consequences of nucleolar insufficiency.
Long-lasting nucleolar transcriptional impairment may prevent the sufficient renewal of ribosomes that are readily oxidized loosing their translational activity (Hetman & Pietrzak, submitted). That may lead to ribosomal failure and reduction in protein synthesis (Honda et al., 2005; Ding et al., 2006). In addition, the diffusion of nucleolus-residing stress response proteins into the nucleoplasm/cytoplasm may trigger chronic activation of stress signaling including accumulation of p53 (Rubbi and Milner, 2003; Emmott and Hiscox, 2009; Boulon et al., 2010).

DNA damage and repair in the nervous system

Age-related onset of neurodegeneration is likely in part a function of the neuronal accumulation of unrepaired DNA damage (Rutten et al., 2003; Barber et al., 2006; Culmsee and Landshamer, 2006; Dolle et al., 2006; Droge and Schipper, 2007; Loerch et al., 2008; Martin, 2008a; McKinnon, 2009). Increased oxidative damage to cell membranes, proteins and nucleic acids, including DNA, has been demonstrated in the most prevalent of the adult-onset neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Ferrante et al., 1997; Gabbita et al., 1998; Zhang et al., 1999; Dizdaroglu et al., 2002; Lovell and Markesbery, 2007a, b).

Due to its high oxygen consumption rate (approximately 20% of consumed oxygen), the brain has an increased susceptibility to oxidative damage compared with other organs. This damage is generated by reactive oxygen species (ROS) derived primarily from the process of oxidative phosphorylation (Lovell and Markesbery, 2007b). Oxidative reactivity to DNA results in the creation of numerous oxidized base adducts, the most prominent of which is 8-hydroxyguanine (8-OHG). ROS can also cause strand breaks and DNA cross links with DNA and proteins (Ferrante et al., 1997; Dizdaroglu et al., 2002). Studies of post-mortem AD patients have identified increased 8-OHG levels in mitochondrial DNA (mtDNA) in the parietal lobe (Mecocci et al., 1993), and in nuclear DNA (nDNA) from the temporal and parietal lobes (Gabbita et al., 1998).

Evidence that such DNA damage triggered DNA damage response has been shown in human diseases and/or animal models of the disease. The accumulation of p53 and presence of activated caspase 3 has been demonstrated in human post-mortem brains

of AD patients, as well as in the brains of transgenic mice overexpressing Aβ. Similarly, oxidative insult resulted in p53-dependent apoptosis in the substantia nigra of mice rendered parkinsonian by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine- (MPTP). Though many studies have demonstrated that cell loss is prominent in the AD, PD, and ALS post-mortem human central nervous system (CNS) (Honig and Rosenberg, 2000), the mechanisms of degenerative changes at the early-stages of these conditions are unknown (Droge and Schipper, 2007). Indeed, apoptosis following DNA damage has been demonstrated in developing neurons and in whole animals, but is attenuated in mature neurons (Lesuisse and Martin, 2002; Martin, 2008a; McKinnon, 2009).

Neurons can accumulate various forms of DNA damage. The repair of oxidative lesions can lead to the release of free bases, or apurinic/apyrimidinic sites (AP). The presence of a lost purine or pyrimidine and the deoxyribose with a cut in the phosphodiester backbone of one strand is a single strand break (SSB), which may also be formed by X-rays, and topoisomerase I inhibiting chemotherapy agents, such as camptothecin (CPT) (Morris and Geller, 1996; McMurray, 2005). Chemical agents, such as the commonly used chemotherapy agents, etoposide and cisplatin, and gamma-irradiation may induce chemical cross-linking of two complementary DNA strands, termed interstrand cross-links (ICLs) and cuts in both strands, termed double strand breaks (DSBs).

Genotoxic lesion repair in post-mitotic neurons employs similar machinery as in other cells. The frequency of genotoxic lesions mediates the activity prominence of the repair pathway. Base excision repair (BER) is the primary repair pathway (Fishel et al., 2007), followed by, nucleotide excision repair (NER) and non-homologous end joining

(NHEJ). Homologous recombination (HR) is believed to be relatively inactive in neurons (McMurray, 2005; Fishel et al., 2007; Lee and McKinnon, 2007). DSB are repaired by NHEJ and HR pathways (McKinnon, 2009). HR is functional only in proliferating cells, whereas NHEJ is active in proliferating and differentiating cells, such as in the mature nervous system. DSB detection is conducted by ataxia telangiectasia, mutated (ATM), ataxia telangiectasia and RAD3-related (ATR), or DNA-dependent protein kinase (DNA-PK) (Hoeijmakers, 2001; McKinnon, 2009). NHEJ is not error-free and proceeds with the recruitment of the subunits of DNA-PK, KU70 and KU86 to the open ends of DNA, which recruits the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs). Without a template to synthesize from, the two broken ends are linked together by ligase 4 (LIG4), which may result in the gain or loss of nucleotides (Hoeijmakers, 2001; McKinnon, 2009).

The signaling of strand breaks involves DNA damage signaling kinases, including poly(ADP-ribose) polymerase I (PARP), ATM, ATR and DNA-PK (Hoeijmakers, 2001; Caldecott, 2008; McKinnon, 2009). ATM is activated by autophosphorylation via interaction with Nijmegen breakage syndrome 1 (NBS1) and the remaining members of the MRN complex (component proteins, MRE11, RAD50) (Goodarzi et al., 2004; Lavin, 2004; Iijima et al., 2008; Morio and Kim, 2008). ATM substrates for a cell salvaging pathway include those responsible for arresting cell cycle and commencing repair (including CHK2 and DNA-PK), whereas substrates of the suicide pathway include p53, caspase 3 and PUMA (Hoeijmakers, 2001; Lee and McKinnon, 2007; Morio and Kim, 2008; McKinnon, 2009). The inhibition of DNA topoisomerases has been shown to activate the DNA damage signaling kinases. Topoisomerase inhibitors, etoposide

(topoisomerase II) (Kruman et al., 2004; Besirli and Johnson, 2006) and camptothecin (topoisomerase I) (Sordet et al., 2009), activate ATM in post-mitotic neurons. The latter study demonstrated this finding in a manner independent of DNA-PK.

Topoisomerases and their chemical inhibitors

The topoisomerases (I and II) catalyze DNA topology rearrangement to maintain its energetically stable forms from the relaxed or super-coiled DNA forms (Sharma and Mondragon, 1995; Bates and Maxwell, 1997). Topoisomerase I regulates DNA topology by cleaving one strand of DNA and creating a covalent 3'-phosphotyrosine bond at its catalytic center (Pommier, 2009). Mammals have two topoisomerase II isoforms, α and β. Topoisomerase IIα is present in proliferating tissues, while IIβ is abundant in terminally differentiated cells (Nur et al., 2007). It is particularly important in neural development, demonstrated by the failure of topoisomerase IIβ knock out mice to establish neuromuscular junctions (Yang et al., 2000) and inhibited neuronal differentiation following its pharmacological inhibition (Tsutsui et al., 2001) (Nur et al., 2007). Topoisomerase II regulates DNA topology by creating a DSB and then passing a double helix through it (Baldwin and Osheroff, 2005). Topoisomerase II forms a covalent 5'-phosphotyrosine bond on both strands of DNA (Pommier, 2009). The DSBs created by topoisomerase II are always protein-bound and thus do not elicit DNA damage responses (Nitiss, 2009a).

The poisoning of topoisomerases bound to DNA has been used in anti-cancer treatments for decades. Camptothecin and etoposide are both derived from plants, *Camptotheca acuminate* (Pommier, 2009) and *Podophyllum peltatum* (Baldwin and

Osheroff, 2005), respectively. The camptothecin derivative topotecan is currently approved for treatments of ovarian cancers and small-cell lung cancers, whereas another derivative, irinotecan, is approved for treatment of colorectal tumors, (Pommier, 2009). Being of planar aromatic structure, camptothecin stacks between the base pairs next to the 3'-phosphotyrosine DNA-protein interface, thereby trapping the topoisomerase I cleavage complex. Once trapped on the DNA of a post-mitotic cell, a transcription blockage can result. Indeed, camptothecin is a potent inhibitor of nucleolar transcription in cancer cell lines (Pommier et al., 2006). The reversible, trapped topoisomerase cleavage complex is removed by tyrosyl-DNA-phosphodiesterase 1 (TDP1) and BER (Pommier, 2006).

Etoposide is also planar and aromatic, yet its precise mechanism of action remains unclear. However, it is known that etoposide acts after topoisomerase II has cleaved DNA, but prior to its relegation (Nitiss, 2009b). Studies have demonstrated that two molecules of etoposide are required to induce double strand breaks in DNA (Bromberg et al., 2003). These findings suggest that, like camptothecin, etoposide inserts itself between the base pairs next to the 5'-phosphotyrosine DNA-protein interface and stabilizes the enzyme on the cleaved DNA. Yet the inhibition of topoisomerase II activity by etoposide has also been demonstrated to produce single strand breaks (Rogojina and Nitiss, 2008). There are numerous etoposide analogs, one significant to the work described herein is meso-2,3-bis(2,6-dioxopiperazin-4-yl)butane (ICRF-193), a topoisomerase II catalytic inhibitor whose activity blocks enzyme turnover by maintaining it in a closed confirmation. ICRF-193 will therefore have less genotoxic potential than etoposide.

Etoposide is one of the most widely used chemotherapy agents, approved by the FDA in 1983 prior to any knowledge that its target was topoisomerase II (Baldwin and Osheroff, 2005). Often in combination with cisplatin, etoposide has been used in the treatment of small-cell lung cancer (Sher et al., 2008), testicular cancer (Feldman et al., 2008), among others, as well as in intra-arterial delivery to the brain for high grade astrocytomas (Madajewicz et al., 2000; Newton, 2005). It is also used for pediatric medullablastoma, primitive neuro-ectodermal tumors, and germ cell tumors. Its use has been extended as a single agent treatment for pediatric oncology involving neuroblastoma, leukemia, Ewing's sarcoma, soft tissue sarcoma, osteogenic sarcoma and Wilms' tumor (Lowis and Newell, 1996). Etoposide has gained clinical attention in the treatment of pediatric high-grade gliomas because of its effectiveness and reduced risk of endocrinological and neurocognitive deficits (Dufour et al., 2006).

Ataxia-telangiectasia mutated (ATM) protein kinase

A member of the phosphatidylinositol 3-kinase (PI3K)-like family of serine/threonine protein kinases (PIKKs), ATM is a 300kD Ser/Thr protein kinase that responds to genotoxic insult. Named for its causal role in the autosomal recessive disorder, Ataxia-telengiectasia (A-T), A-T mutated protein kinase (ATM) was originally identified following reduced inhibition of DNA synthesis in response to increasing doses of ionizing radiation (Houldsworth and Lavin, 1980; Painter and Young, 1980; Lavin, 2008). ATM mediates the signaling of DNA double stranded breaks (DSB) to numerous downstream targets, including BRCA1, p53, Chk2, CDK2, and H2AX in order to facilitate a range of cellular processes that include DNA repair and cell cycle control.

The activated downstream signaling of ATM has been observed to be required for the cellular response to genotoxic insult (irradiation and etoposide treatment) in the nucleolus (Kruhlak et al., 2007), as ATM absence resulted in continued Pol1-mediated transcriptional activity.

To successfully transduce the message of genotoxic damage, ATM must itself be activated. This activation first involves the monomerization of ATM from its inactive dimer form, and subsequent activation by autophosphorylation and acetylation (Bakkenist and Kastan, 2003; Sun et al., 2007; Sun et al., 2009; Kozlov et al., 2011). Autophosphorylations at S1981, S1893 and S367 are well-characterized and mutations of these sites in A-T cells are unable to sufficiently correct radiation-induced cell-cycle defects (Bakkenist and Kastan, 2003; Kozlov et al., 2006). Interestingly, bacterial artificial chromosome (BAC) constructs using serines mutated to alanines on these autophosphorylation sites were introduced into an $Atm^{-/-}$ background and ATM autophosphorylation and downstream signaling was normal (Pellegrini et al., 2006; Daniel et al., 2008). These findings prompted the identification of additional DNA damage-induced ATM autophosphorylation sites (Kozlov et al., 2011).

Recently the identification of ATM activity in its "inactive" dimerized state provided evidence of a novel DNA repair function. Hydrogen peroxide caused the activation of the dimerized form of ATM to phosphorylate a substrate, the N-terminus of a glutathione S-transferase (GST)-p53 in HEK293T cells *in vitro* (Guo et al., 2010a; Guo et al., 2010b) in the absence of DSBs. The authors later confirmed that the FRAP/ATM/TRRAP C-terminal domain (FATC) was responsible for the oxidative damage signaling of ATM. A mutation of cysteine residue 2991 allows DSB-induced

ATM signaling, but is unresponsive to H_2O_2. Although autophosphorylation at S1981 was not increased, no other DNA damage-mediated autophosphorylation sites were evaluated (Guo et al., 2010a; Guo et al., 2010b). The importance of these findings is three-fold: first, a subset of A-T patients, termed A-T variants, exhibit only moderate radiosensitivity and no immunodeficiency, yet still acquire the primary neurological component of A-T, ataxia. This mutation results from the entire lack of the last 10 amino acids on the C-terminus. The protein made from this mutation, R3047X, is fully activated by the MRN complex, but is not activated by oxidation in vitro (Guo et al., 2010b). Second, a novel ATM activation pathway has been established with an activating stimulus that was anticipated, based upon decreased lymphoma incidence and the reduced loss of hematopoietic stem cells in $Atm^{-/-}$ mice treated with antioxidants (Ito et al., 2004; Reliene and Schiestl, 2007; Reliene et al., 2008). Lastly, the dimeric form of ATM accomplishes the signaling mediated by the oxidative stress stimulus (Guo et al., 2010a; Guo et al., 2010b).

ATM: more than a DNA damage signaling molecule

During the last decade publications have arisen sporadically, which implicate ATM in non-DNA damage response-related activities. These studies have been prompted by the varied phenotypic consequences of an insufficiency in ATM, as described by the autosomal recessive disorder, A-T. A-T patients suffer from high incidences of type 2 diabetes mellitus, progeria, telangiectasias, and neurological phenotypes that include cerebellar ataxia and speech defects, as well as the expected increased cancer incidence and radiosensitivity (McKinnon, 2004; Lavin, 2008). Thus, it has been shown that ATM

is activated by insulin to stimulate translation via the direct phosphorylation of 4E-BP1, which then triggers the phosphorylation-dependent release of 4E-BP1 from eIF-4E (Yang and Kastan, 2000). These studies have been followed up to show that ATM mediates the activity of AKT to promote survival, without which apoptosis occurs in cycling cells (Halaby et al., 2008; Li and Yang, 2010).

The origin of the neurological phenotype associated with A-T is yet unknown. Recent work has identified mild to severe cognitive impairments in A-T patients, consistent with A-T Like Disorders (ATLD). ATLD is rooted in the impaired function of MRE11, Rad50, or NBS1 genes, who respectively code for the proteins that make up the MRN complex. However, unlike ATLD, the A-T neurological component does not include microcephaly, but rather cerebellar atrophy and language impairment was restricted to oral motor impairments (Vinck et al., 2011). As the most noticeable deficit is ataxia that usually begins in the second decade of life, it is logical that the identified speech deficits would be motor-based. The most common theories of the neurophenotype origin include the inability of neurons to signal damage (oxidative or DSB) to be repaired.

Neuronal ATM is found in the nucleolus, the nucleoplasm and in the cytoplasm (Gorodetsky et al., 2007). Recent work has indicated that the cytoplasmic form of ATM signals to synapsin-1, a vesicle component, to regulate neurotransmitter release (Li et al., 2009), the absence of which has profound effects on hippocampal long-term potentiation in mice. The implications of this work include the novel function of ATM, its residence-specific function, and the likely contribution to the neurological component of A-T. While the nucleolar enrichment of ATM prompted speculations about its possible role in

maintaining the high rate of nucleolar transcription, perhaps by repairing transcription related DNA damage (Gorodetsky et al., 2007), experiments testing such a notion have not yet been reported.

Targets promoting sufficient neuronal nucleolar transcription

Collectively, nucleolar transcription appears critical for neurons in regulating their growth. In addition, by ribosomal renewal and stress sensing including monitoring of genomic integrity, the nucleolus plays an essential role in neuronal maintenance. Finally, the nucleolus appears to be a target of neurodegeneration. However, regulation of nucleolar transcription in neurons remains poorly understood. In particular, when this work began it had not been known whether DNA damage could disrupt neuronal nucleolar transcription in the intact animal brain and whether such a disruption may occur in mature adult neurons. Additionally, no signaling pathway other than ERK1/2 had been implicated as a regulator of neuronal Pol1. This study took advantage of the topoisomerase-II poison, etoposide, to test the hypothesis that nucleolar transcription is sensitive to DNA damage in whole animal brains and that such sensitivity is not age-restricted. In addition, we followed accidental observations suggesting ATM's involvement in Pol1 regulation and tested the hypothesis that this kinase stimulates neuronal Pol1 transcription while regulating neuronal growth upon neurotrophic stimulation.

CHAPTER II

NUCLEOLAR DISRUPTION AND APOPTOSIS ARE DISTINCT NEURONAL RESPONSES TO ETOPOSIDE-INDUCED DNA DAMAGE[a]

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Introduction

Oxidative damage to neuronal DNA, including chemical modifications of DNA bases and single strand breaks (SSBs), is well-documented in several age-dependent neurodegenerative conditions including Alzheimer's- or Parkinson's diseases (Markesbery and Lovell, 2006; Martin, 2008a). In addition, the accumulation of oxidative DNA damage in neurons has been demonstrated during brain aging (Brasnjevic et al., 2008; Yankner et al., 2008). However, it is unclear whether such genomic injuries contribute to age-related neurodegeneration or represent an epiphenomenon of oxidative stress. Therefore, it is critical to test whether the neurodegeneration-associated forms of DNA damage result in neurotoxic consequences in the adult or aging brain.

Studies of neurons from embryonic and/or neonatal rodents have identified apoptotic cell death as a major neuronal response to DNA damage. Neuronal apoptosis has been observed following challenges with DNA strand break inducers, such as

topoisomerase inhibitors or ionizing irradiation (Enokido et al., 1996; Morris and Geller, 1996; Herzog et al., 1998; Martin et al., 2009). However, that effect is limited to relatively immature neurons (Herzog et al., 1998; Martin et al., 2009). Therefore, a non-apoptotic response to neuronal DNA damage may be more relevant for the pathogenesis of age-dependent neurodegenerative diseases.

Genetic defects of several DNA repair pathways, including those for single- or double DNA strand breaks (SSBs or DSBs, respectively) as well as large chemical modifications of DNA bases that are also known as 'bulky' DNA adducts (Brooks, 2008; Caldecott, 2008; Katyal and McKinnon, 2008) provide a strong argument for a causative role of DNA damage in common neurodegenerative disorders. For instance, deficiencies of the DSB repair kinase ataxia telangiectasia-mutated protein kinase (ATM) trigger delayed neurodegeneration, presumably by blocking the developmental apoptosis of newly generated neurons harboring chromosomal abnormalities (Katyal and McKinnon, 2008). In contrast, the SSB/bulky adducts repair deficits may induce neurodegeneration by the progressive and age-dependent accumulation of unrepaired DNA damage that reduces neuronal transcription (Brooks, 2008; Caldecott, 2008; Katyal and McKinnon, 2008). In the nervous system of whole animals, evidence for linking such genomic lesions to transcriptional insufficiency has not yet been reported.

The nucleolus is a site of ribosomal biogenesis (Grummt, 2003; Drygin et al., 2010). That process is initiated by the RNA-Polymerase-1 (Pol1)-driven transcription of nucleolar rRNA genes. Continuous Pol1 activity is required for maintaining nucleolar structure. Consequently, Pol1 inhibition results in nucleolar disruption, which triggers the release of various proteins whose localization is normally restricted to nucleoli. Upon

release from the nucleolus some of those proteins lead to the activation of the pro-apoptotic p53 pathway (Rubbi and Milner, 2003; Yuan et al., 2005). In proliferating cells, this mechanism has been proposed to underlie the cytotoxic effects of several DNA damaging anti-cancer drugs (Rubbi and Milner, 2003; Burger et al., 2010). Work from our laboratory has demonstrated that in cultured cortical neurons from newborn rats, the DNA damaging DNA-Topoisomerase-1 (Topo1) inhibitor camptothecin results in Pol1 inhibition and nucleolar stress, which is sufficient to induce p53-dependent apoptosis (Kalita et al., 2008). Hence, nucleolar transcription may serve as a sensor of neuronal DNA damage including such camptothecin-induced DNA lesions as SSBs or Topo1 cross-links to DNA. The consequences of activating such a sensing mechanism could be p53-mediated apoptosis or a chronic reduction of ribosomal biogenesis if the apoptotic response is disabled.

Etoposide is a widely-used anti-cancer drug that, by inhibiting DNA-topoisomerase-2 (Topo2), induces DSBs, SSBs and Topo2-DNA cross-links (Nitiss, 2009b). In humans, etoposide-containing multidrug chemotherapy has been associated with mild cognitive impairment in children and adults (Kaasa et al., 1988; Mok et al., 2005; Whitney et al., 2008; Riva et al., 2009). In adult rats, acute neurotoxicity has been reported following intracarotid etoposide injections that were combined with the osmotic disruption of the blood–brain barrier (BBB) using mannitol (Maeda et al., 1999; Fortin et al., 2007). To date, there have been no published studies investigating mechanisms of etoposide-induced neurotoxicity in whole animals. The current study was performed to determine whether nucleolar stress occurs following etoposide-mediated neuronal DNA damage and whether such a response is developmentally-restricted.

Materials and Methods

Animals

Sprague–Dawley rats were purchased from Harlan (Indianapolis, IN, USA). All animal experiments strictly followed the protocols that were approved by the Institutional Animal Care and Use Committee of the University of Louisville and the NIH guidelines.

Materials

The following antibodies and reagents were obtained from commercial sources: anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti- (phospho-Ser15)-p53 (Cell Signaling Technology, Danvers, MA, USA); anti-B23 (mouse monoclonal; Santa Cruz Biotechnology); anti-(Asp175)-cleaved caspase 3 (rabbit polyclonal; Cell Signaling Technology); anti-bromo-deoxyuridine (anti-BrdU, mouse monoclonal; Sigma, St Louis, MO, USA); anti-β-galactosidase (rabbit polyclonal anti-β-gal; MP Biomedicals, Solon, OH, USA); anti-HA (mouse monoclonal; Roche Diagnostics, Indianapolis, IN, USA), anti-β-actin (mouse monoclonal; Sigma); anti-microtubule-associated protein 2 (MAP2) (mouse monoclonal; Cell Signaling Technology), and anti-phospho-Ser1981-ATM (mouse monoclonal; Cell Signaling Technology); horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (Calbiochem, San Diego, CA, USA); Alexa 488-, Alexa 594-conjugated secondary antibodies, and, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA); camptothecin, etoposide, ICRF-193, 5-fluorouridine (5FU); 4-hydroxytamoxifen (4HT) (Sigma). Respective IgG antibodies were used in place of primary antibodies as a negative control (data not shown).

Intracarotid artery injections of etoposide

Young adult male rats (8–10 weeks old, 200–250 g) were deeply anesthetized with an intramuscular injection of 3.3 mL/kg mixture containing 25 mg/mL ketamine hydrochloride (Abbott Laboratories, Abbott Park, IL, USA), 1.2 mg/mL acepromazine maleate (The Butler Company, Dublin, OH, USA), and 0.25 mg/mL xylazine (The Butler Company) in 0.9% saline. A midline incision on the neck, longitudinally, to the jugular notch was then made. Blunt dissection through the fat pad to separate the sterno-hyoid from sterno-mastoid muscles proceeded. An alm retractor was applied to separate the omo-hyoid and sterno-mastoid muscles. The omo-hyoid was lifted, cut and reflected laterally. The common carotid was carefully dissected from the vagus and sympathetic nerves using a #7 Dumont forceps. A 6.0 size suture was placed under the most caudal aspect of the common carotid artery and lifted to temporarily occlude it. A 30 gauge needle attached to a syringe via a catheter was inserted into the carotid just rostral to the occlusion for injection of 20%D-mannitol and 15 mg/kg etoposide. Three milliliters of mannitol was infused over 30 s followed by a 5-min pause prior to the slow infusion of 1 mL etoposide or 20% dimethylsulfoxide (DMSO) in 0.9% saline. After completion of the injection, a microaneurysm clip was applied to the most rostral aspect of the exposed common carotid artery. Quick stop powder was applied to quickly promote coagulation and the suture was removed. The microaneurysm clip was removed prior to closing the incision with wound clips.

Intracerebroventricular injection

Rat pups received injections at postnatal day 7 (P7) based on a previously described method (Han and Holtzman, 2000). The injections of 5 μL artificial CSF (126 mM NaCl, 25 mM $NaHCO_3$, 25 mM glucose, 1.25 mM NaH_2PO_4, 2.5 mM KCl, 1 mM MgCl) / 20%DMSO ± 10 nmoles etoposide were made with a Hamilton needle inserted into the left lateral ventricle at the following coordinates: 1.5 mm rostral- and 1.5 mm lateral to lambda (incorrectly named Bregma in (Han and Holtzman, 2000), Dr David Holtzman, personal communication), 2 mm deep from the skull surface. Lambda was readily identified by the underlying venous sinuses which were visualized by shining light from two focal sources placed on either side of the head (D. Holtzman, personal communication). After injection, animals were placed back with their mother.

Cell culture and transfection

Cortical neurons were isolated from newborn Sprague–Dawley rats at postnatal day 0 as described previously (Habas et al., 2006). Neurons were cultured in basal medium Eagle supplemented with 10% heat-inactivated bovine calf serum (Hyclone, Logan, UT, USA), 35 mM glucose, 1 mM L-glutamine, 100 U/mL of penicillin and 0.1 mg/mL streptomycin or Neurobasal A/B27 medium (Invitrogen). On day *in vitro* 2 (DIV2), 2.5 μM cytosine arabinoside was added to cultures to inhibit the proliferation of non-neuronal cells. Cells were used for experiments on DIV5–6 unless indicated otherwise.

Drug treatment of cultured neurons

For treating cultured cells, etoposide, camptothecin, ICRF-193 or 4HT were dissolved in DMSO or methanol, respectively. The final media concentration of the solvents was 0.2%.

RNA isolation and quantitative RT-PCR

Following CO_2 euthanasia, the entire rat neocortex was harvested and immediately frozen on dry ice. Tissue RNA extraction was done following homogenization with a pestle grinder (Fisherbrand; Fisher Scientific, Pittsburgh, PA, USA) in 1 mL TRI Reagent (Sigma) at 4°C. Cultured cell RNA was isolated from 5×10^6 cells/sample using TRI Reagent. The random hexamers-primed cDNA synthesis, quantitative RT-PCR, and the ΔΔct-based analysis of 45S pre-rRNA/18S rRNA levels were done as described previously (Kalita et al., 2008).

Immunofluorescence

Deeply anaesthetized rats were transcardially perfused with ice cold PBS, and then with 4% paraformaldehyde in PBS. Following 24 h post-fixation, brains were cryoprotected for up to 72 h in 30% sucrose and sectioned on a cryostat at 25 μm sections (P7 rats) or 30 μm sections (adult rats), and mounted on slides. For B23 staining, epitope rescue was carried out in 10 mM sodium citrate buffer, 0.05%Tween, pH 6.0 for 20 min at 90°C. Then, sections were incubated in blocking solution [3% bovine serum albumin, 10% normal goat serum in PBS/0.1% Triton-X100 (PBST), 1 h] followed by primary and

secondary antibodies (anti-B23, 1:100, 48 h at 4°C and anti-mouse-IgG-Alexa-488, 1:100, 4 h at 20°C, respectively). Activated caspase 3 (Asp175) immunostaining was performed following a standard protocol. Briefly, after 1 h blocking (3% bovine serum albumin, 5% normal goat serum in PBST), sections were incubated with an antibody specific for the cleaved, activated caspase 3 (1 : 100, over night at 4°C) followed by an Alexa-488-labled secondary antibody (1 : 100, 1 h at 20°C). Nuclei were visualized by Hoescht-33258. Then, sections were covered with coverslips that were mounted using Fluoromount G (Southern Biotech, Birmingham, AL, USA).

Image acquisition and analysis

Images were captured using the Nikon Eclipse TE2000-S or the Zeiss AxioObserver inverted microscopes with MetaMorph or AxioVision software, respectively. Fluorescent exposure rates were kept consistent across all conditions by fluorescent filter. Images were exported as TIF files with and without scale bar annotations. Both the annotated and unannotated TIF images were further scaled in Photoshop7 (Adobe Systems Incorporated, San Jose, CA, USA) and a line of appropriate font was applied to the unannotated TIF that matched the size of the annotated TIF in Adobe Illustrator 10 (Adobe).

Evaluation of apoptosis

Apoptosis was evaluated by nuclear morphology. Cells were stained with Hoechst 33258 and anti-cleaved caspase-3 observed using fluorescent microscopy. Cells

with condensed or fragmented nuclei were scored as apoptotic. Though quantifications were not provided in figures shown in this thesis, *in vitro* quantification provided in the published work were conducted by counting both Hoechst 33258-stained apoptotic bodies and live nuclei. Total apoptotic nuclei were divided by the summed total of counted nuclei (healthy and apoptotic) to determine the apoptotic ratio. No less than 300 cells were counted and all areas of the stained coverslip were included in the calculations.

Statistical analysis

Statistical analysis of the data was performed using one-way ANOVA. Error bars were expressed as standard errors of the mean.

Results

Nucleolar stress in cortical neurons of etoposide-treated rats

To investigate the nucleolar consequences of etoposide-induced DNA damage in adult rats, we performed intracarotid injections of 15 mg/kg etoposide immediately after osmotic BBB disruption with 20% mannitol. Although such delivery may reach various parts of the brain, we focused our analysis on the neocortex. Four hours after etoposide-injection, the neocortical 45S pre-rRNA/18S rRNA ratio decreased to 0.51-fold of vehicle-treated controls (Fig. 4A, $p < 0.05$). As 45S pre-rRNA is the primary Pol1 transcript that is rapidly processed to mature rRNA species, including 18S rRNA, the declining 45S/18S ratio indicates Pol1 inhibition (Kalita et al., 2008). This conclusion is further supported by the immunostaining for the nucleolar marker B23/nucleophosmin

(Fig. 4B), as nucleolar localization of that protein depends on active Pol1 (Rubbi and Milner, 2003; Kalita et al., 2008). Hence, upon Pol1 inhibition, B23 is released from the nucleoli and diffuses throughout the nucleoplasm (Rubbi and Milner, 2003; Kalita et al., 2008). In vehicle-, but not etoposide-injected rats, B23 immunostaining was concentrated in the nucleoli of neocortical cells (Fig. 4B). Six hours after etoposide treatment, B23 immunostaining became uniformly distributed in the nucleoplasm of those cells, suggesting that they are under nucleolar stress (Fig. 4B). The nucleolar stress was present in virtually all neocortical cells (Fig. 4B, and data not shown). Hence, in cortical neurons of adult rats, nucleolar disruption is a response to the DNA damaging drug etoposide.

To determine whether such a response is also present in the developing brain, 7-day old rats were administered 10 nmoles of etoposide by a single injection into the right lateral ventricle. After 4 h, the declining ratio of 45S pre-rRNA/18S rRNA indicated Pol1 inhibition in the ipsilateral neocortex of etoposide-treated animals (Fig. 5A). In addition, etoposide induced a partial release of nucleolar B23 in almost all analyzed cells from the ispilateral neocortex (Fig. 5B). Therefore, the etoposide-induced nucleolar stress response occurs both in developing and mature cortical neurons of whole rats.

Figure 4

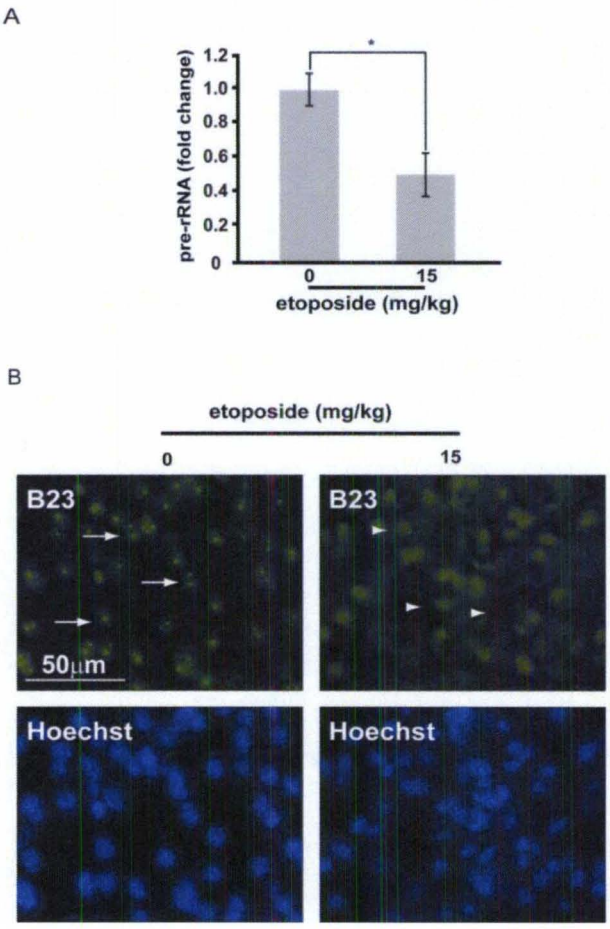

Figure 4: Nucleolar disruption in the neocortex of etoposide-treated adult rats. Rats were infused with 0 or 15 mg/kg etoposide that was administered into the right intracarotid artery immediately after BBB disruption with 20% d-mannitol. (A) At 4 h after etoposide treatment, reduced nucleolar transcription was observed in the ipsilateral neocortex as indicated by the declining expression ratio of the primary nucleolar rRNA transcript (45S pre-rRNA) to the mature 18S rRNA. The rRNA levels were determined by quantitative real-time PCR; data are mean ± SE of four animals per group; $*p < 0.05$. (B) At 6 h after etoposide treatment, nucleolar stress was observed throughout the ipsilateral neocortex as indicated by the loss of nucleolar immunofluorescence of nucleolphosmin/B23. B23, which under control conditions was concentrated in nucleoli (arrows), displayed a diffused staining pattern throughout the nucleoplasm in etoposide-infused rats (arrowheads). Note that cells with nucleolar stress had normal chromatin structure as visualized by counter-staining with Hoechst-33258. Representative

photomicrographs of cortical layer IV–VI are shown; similar effects of etoposide on B23 localization were observed in three animals.

Figure 5

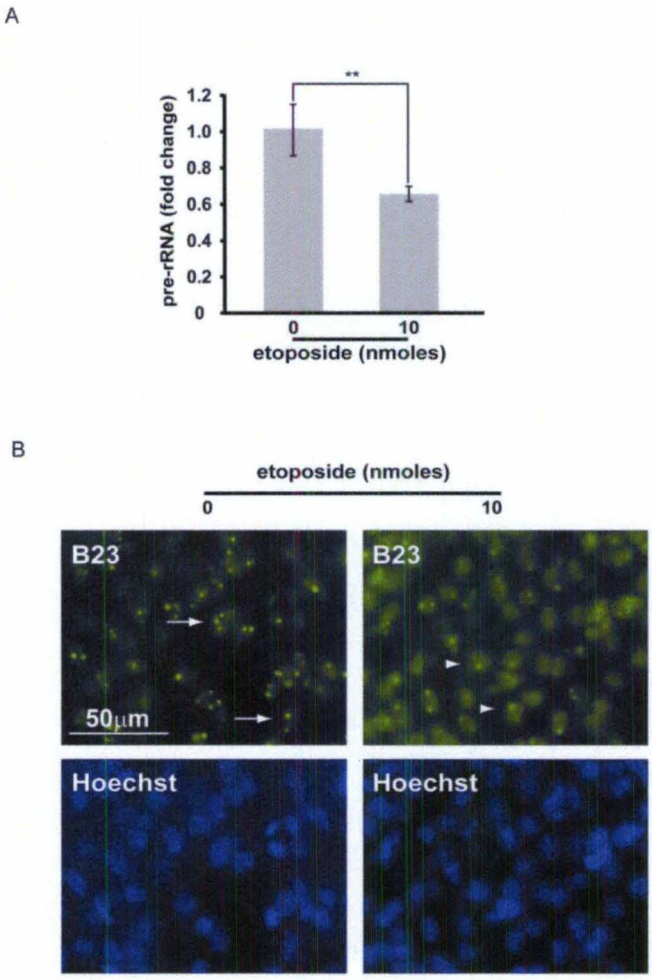

Figure 5: Nucleolar disruption in the neocortex of etoposide-treated newborn rats. At postnatal day 7 (P7), rats received 0 or 10 nmoles etoposide that was administered by an injection into the right lateral ventricle. (A) Four hours after etoposide injection, nucleolar transcription was suppressed in the ipsilateral neocortex. Data are means ± SE of four animals per group; $^{**}p < 0.01$. (B) Six hours after etoposide injection, nucleolar stress was present in the ipsilateral neocortex. Reduced nucleolar B23 staining together with its diffused appearance throughout the nucleoplasm was observed in most neocortical cells (arrowheads). B23 under control conditions was concentrated in nucleoli (arrows). Note that cells with nucleolar stress had normal chromatin structure as visualized by counter-staining with Hoechst-33258. Representative photomicrographs of cortical layer IV–VI are shown; similar effects of etoposide on B23 localization were

observed in four animals.

Etoposide-induced apoptosis is developmentally-restricted

Conversely, etoposide triggered apoptosis in developing, but not mature cortical neurons. In the neocortex of vehicle-injected neonates, immunostaining for the activated form of caspase 3 revealed rare apoptotic cells (up to 2–3 cells/section) (Fig. 6A-C, and data not shown). In contrast, numerous cells with active caspase 3 were observed in the neocortex of etoposide-injected neonates at 6 h post-injection (Fig. 6D, F, H). Such cells were most frequent in the proximity of the injection site and spread throughout all cortical layers. In addition to active caspase 3, they often displayed apoptotic chromatin condensation (Fig. 6E, G). The morphology of some active caspase 3-positive cells revealed neuronal features, including pyramidal perikaria and neurites (Fig. 6D, F). In addition, cells with apoptotic rounding of the shrunken cell bodies were also present (Fig. 6H). Although most apoptotic cells were identified in the ipsilateral neocortex, their increased incidence was also noticed on the contralateral side (data not shown). After 24 h, few apoptotic cells were present and tissue destruction was observed around the etoposide-, but not the vehicle injection site (data not shown). Neither caspase 3 activation nor apoptotic chromatin condensation was present in the neocortex of etoposide- or vehicle- treated adult rats (Fig. 6I-N).

Figure 6

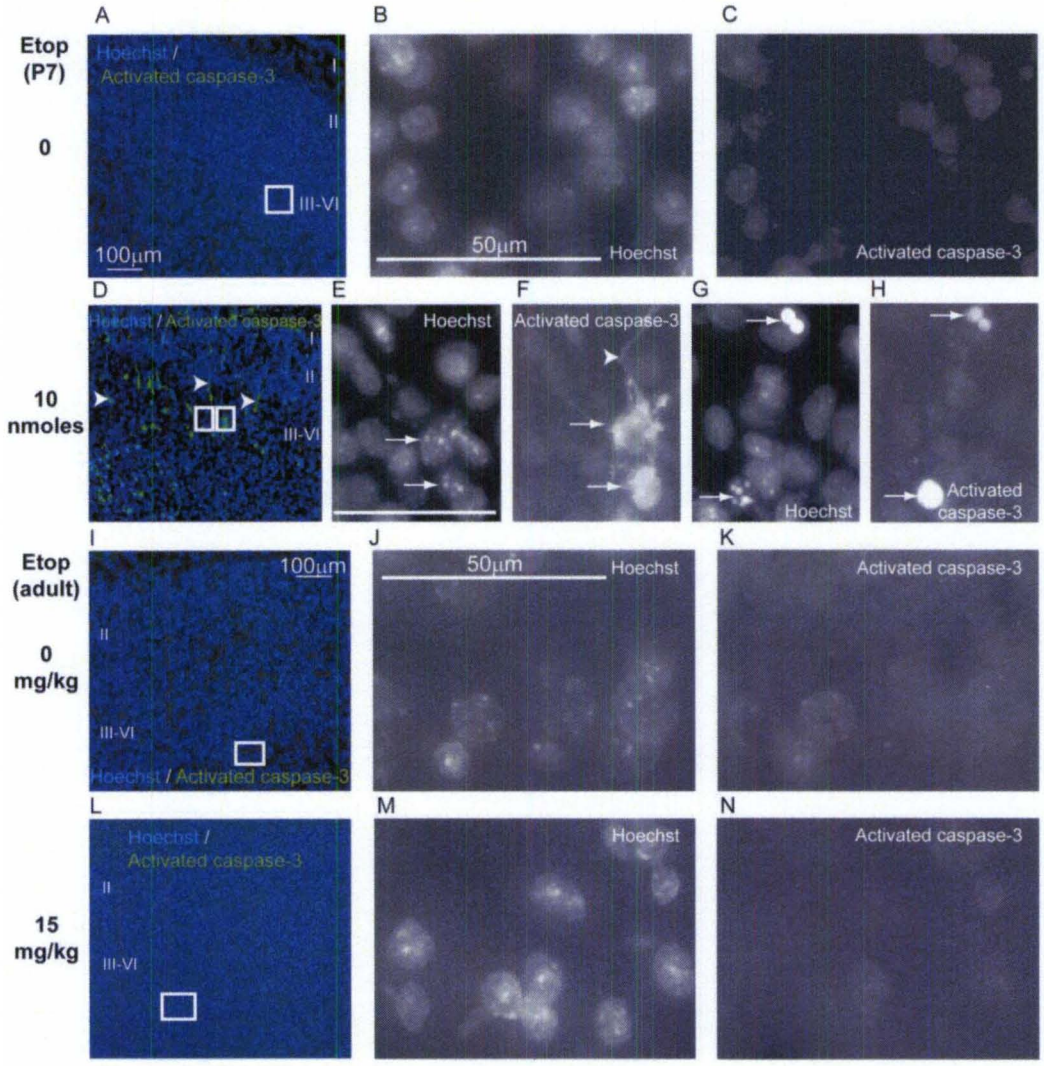

Figure 6: Etoposide-induced apoptosis in the neocortex of the neonate, but not the adult rats.

Neonate (P7) (A–H) or adult rats (I–N) were treated as described for Figs 5 and 4, respectively. Six hours after etoposide (Etop) administration apoptosis was identified by the presence of immunofluorescence for activated caspase 3 and/or condensation/fragmentation of nuclear chromatin that was visualized by counter-staining with Hoechst-33258. Photomicrographs of the ipsilateral neocortex are shown. Three animals were analyzed for each condition. Cortical layers are indicated by roman numerals. (A–H) Activated caspase 3 and apoptotic chromatin rearrangements in neocortical cells of etoposide-treated neonates (arrows). Presence of active caspase 3 in

neurites suggests neuronal identity of many apoptotic cells (arrowheads). (I–N) Absence of apoptotic cells from the neocortex of etoposide-treated adult rats.

The pronounced apoptotic response was also present in the hippocampi of the etoposide-treated neonatal rats (Fig. 7D-F). Apoptotic cells were most frequent in the granule cell layer of the ipsilateral dentate gyrus (Fig. 7D-F). Increased frequency of such cells was also observed on the contralateral side (data not shown). Finally, some caspase 3-positive cells appeared in the pyramidal cell layer of the ipsilateral hippocampus (data not shown). As in the neocortex, no evidence for etoposide-induced apoptosis was found in the adult hippocampi or other analyzed structures of the adult rat brain (Fig. 7G-L, and data not shown). Therefore, unlike nucleolar stress, apoptosis is a developmentally restricted response to etoposide-induced DNA damage.

Figure 7

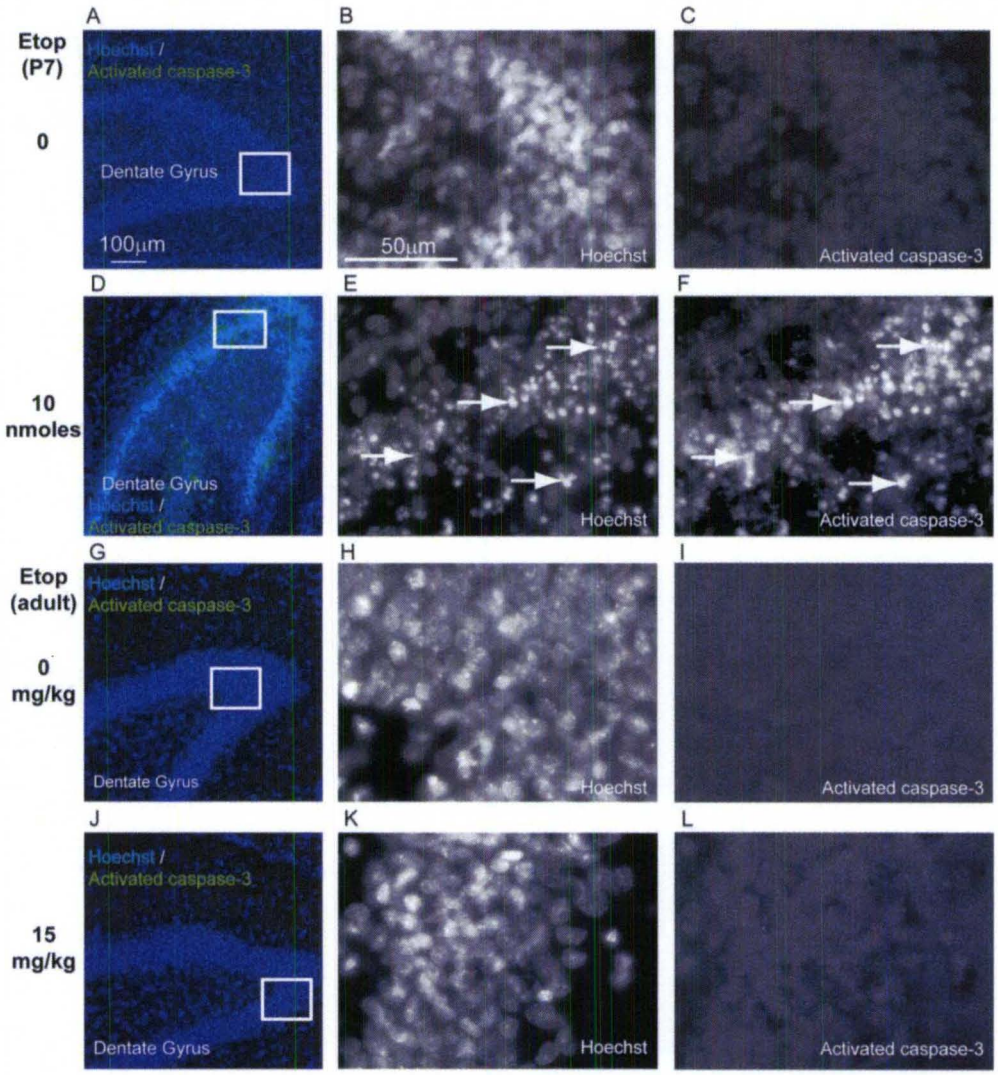

Figure 7: Etoposide-induced apoptosis in the hippocampus of the neonate, but not the adult rats.

Neonate (P7) (A–F) or adult rats (G–L) were treated as described for Figs. 5 and 4, respectively. Six hours after etoposide (Etop) administration apoptosis was identified as in Fig. 6. Photomicrographs of the ipsilateral hippocampi are shown. Three animals were analyzed for each condition. (A-F) Etoposide-induced activation of caspase 3, and apoptotic chromatin rearrangements in granule cells of the neonate dentate gyrus (arrows). (G-L) Absence of apoptotic cells from the dentate gyrus of etoposide-treated adult rats.

Etoposide-induced damage of neuronal DNA causes nucleolar disruption

As in whole animal studies, the neuronal effects of a drug may be indirect, cultured cortical neurons from newborn rats were used to test whether nucleolar disruption is caused by etoposide-induced damage of neuronal DNA. In neurons that were incubated for 8 h with etoposide, the 45S/18S rRNA ratio decreased in an etoposide concentration-dependent manner (Fig. 8). Declines to 0.64- or 0.55-fold of vehicle-treated controls were triggered with 25- or 50 μM etoposide, respectively, indicating Pol I inhibition. In contrast, 10 μM etoposide did not affect the 45S/18S rRNA ratio.

Figure 8

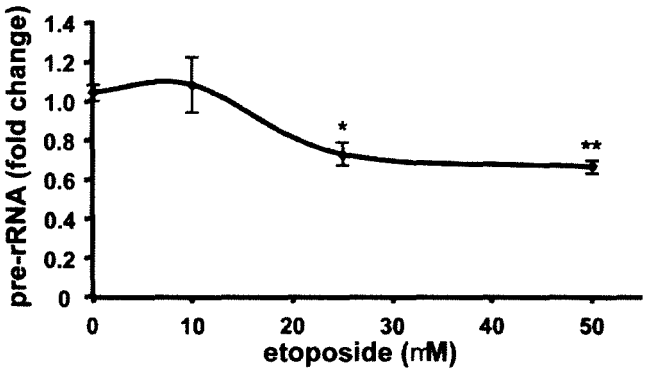

Figure 8: Etoposide-induced nucleolar stress in cultured cortical neurons from newborn rats.

At DIV5, rat cortical neurons were treated with etoposide for 8 h as indicated. At etoposide concentrations of 25- or 50- but not 10 μM, the ratios of 45S pre-rRNA/18S rRNA levels declined, suggesting inhibition of Pol1-mediated transcription. Data represents mean ± SE of three independent experiments; $*p < 0.05$; $**p < 0.01$.

Discussion

In this study, we obtained evidence that the DNA damaging drug etoposide inhibits Pol1-mediated transcription triggering nucleolar stress in both developing and mature cortical neurons of whole rats. That contrasts with neuronal apoptosis, whose induction by etoposide appears developmentally-restricted. Hence, we propose that nucleolar disruption is a DNA damage response that occurs in both young and adult neurons and, as such, may contribute to neurotoxic consequences DNA strand breaks that are associated with neurodegenerative diseases.

Etoposide was used to investigate the nucleolar effects of neuronal DNA damage in whole animals. For adult rat studies, we applied that agent by intracarotid injection that was performed immediately after osmotic disruption of the BBB. That method was chosen because of its demonstrated effectiveness in delivering etoposide to the adult animal- or human brain (Maeda et al., 1999; Fortin et al., 2007). Using that technique, we showed nucleolar disruption, but no apoptosis in the ipsilateral cortices of rats receiving 15 mg/kg etoposide. By 5 h after injecting such a dose, all animals displayed signs of systemic drug toxicity including hypoactivity and slowed breathing. Therefore, it is possible that the disruption of nucleolar B23 at 6 h after treatment may be a secondary response to such toxicity. However, Pol1 inhibition was also observed in all animals at 4 h post-injection when the first signs of systemic toxicity were present in five of eight treated rats. In addition, nucleolar stress was induced in cortical neurons of young animals that received etoposide by intracerebroventricular injection and remained in excellent health for at least 24 h post-treatment. Finally, in cultured cortical neurons that were directly exposed to etoposide, nucleolar disruption was present. Therefore, the

etoposide effects on the nucleolar integrity of cortical neurons are likely associated with its direct genotoxic actions in those cells. Indeed, in the developing rat neocortex, we observed etoposide-induced nucleolar stress and apoptosis without any signs of major systemic toxicity. Hence, intracerebroventricular injection of etoposide provides a convenient model to investigate consequences of neuronal DNA damage in whole rat pups.

As excessive accumulation of DNA strand breaks is associated with neuronal dysfunction/neurodegeneration. Nucleolar stress may be among the neurotoxic effector mechanisms that are triggered by such genotoxic lesions. While in common neurodegenerative diseases the genotoxic frequency will unlikely reach the levels that will be needed for a complete block of Pol1-driven transcription and the consequent disruption of the nucleolar structure, they may reduce ribosomal biogenesis to such levels that will be insufficient to maintain fully functional protein synthesis machinery, thereby leading to neuronal atrophy including loss of synapses. Of note, reduced ribosomal biogenesis is suggested in the Alzheimer's Disease-associated cerebro-cortical pathology that also involves oxidative DNA damage (Ding et al., 2005; Honda et al., 2005; Markesbery and Lovell, 2006). Therefore, DNA damage-induced nucleolar stress may contribute to Alzheimer's Disease.

In conclusion, our results demonstrate that Pol1 inhibition is a neuronal response to DNA damage that is not developmentally restricted, while neuronal apoptosis is a developmentally-restricted DNA damage response. Hence, nucleolar stress may be one of the neurotoxic effector mechanisms of DNA damage in the adult/aging brain.

Acknowledgements

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CHAPTER III

REGULATION OF RNA POLYMERASE-I-MEDIATED TRANSCRIPTION BY ATAXIA TELEANGIECTASIA MUTATED (ATM)

Introduction

Ataxia-telangiectasia mutated (ATM) is a protein kinase that belongs to the PI3K family including such members as mTOR, PI3K, DNA-PK, ATR and SMG-1. ATM is activated by DNA double-stranded break (DSB) damage, thereby playing an important role in the DNA damage response (Herzog et al., 1998; Gatti et al., 2001; Biton et al., 2008; Lavin, 2008). Its absence or insufficiency causes the autosomal recessive disorder Ataxia-telangiectasia (A-T), which is characterized by cancer predisposition, immunodeficiency, and neurodegeneration leading to cerebellar ataxia (Lavin and Shiloh, 1997; Gatti et al., 2001; McKinnon, 2004). The A-T associated neurodegeneration includes Purkinje and granule cell loss in the cerebellum and may also be present in other brain structures such as forebrain, brain stem and spinal cord (Lavin and Shiloh, 1997; Biton et al., 2008; Li et al., 2011). The mechanisms of neurodegeneration in A-T patients remain unknown.

There are several functions of ATM that appear unrelated to its involvement in the DNA damage response. In addition to mTOR, ATM directly phosphorylates 4E-BP1 increasing translation in insulin-stimulated cells (Yang and Kastan, 2000). Similarly

ATM has been shown to activate AKT to promote cellular survival (Li and Yang, 2010). The CREB-dependent differentiation of human SH-SY5Y neuroblastoma cells also requires ATM (Fernandes et al., 2007). At the synapse, ATM regulates neurotransmitter release, contributing to synaptic plasticity (Li et al., 2009). Such multi-functionality of ATM is supported by its widespread distribution throughout the cell including the nucleus, nucleolus, vesicles and cytoplasm (Gorodetsky et al., 2007; Lavin, 2008; Li et al., 2009). Indeed, the functional versatility of ATM may explain the complex pathology of A-T.

The nucleolus is a transcription-dependent structure that contains clustered repeats of the 45S rRNA genes (ribosomal DNA, rDNA). The transcription of rDNA by RNA Polymerase-I (Pol1) is the first step of ribosomal biogenesis (Grummt, 2003; Drygin et al., 2010). By controlling ribosomal biogenesis, Pol1 activity determines cell proliferation and cell growth. Therefore, Pol1 is tightly regulated to adjust ribosomal biogenesis to cellular needs and resources (Grummt, 2003). Indeed, cellular growth, including neurite outgrowth, requires growth factor-dependent activation of Pol1 that is mediated by ERK1/2- and mTOR signaling pathways (Grummt, 2003; Mayer et al., 2004; Drygin et al., 2010; Gomes et al., 2011). Conversely, Pol1 is inhibited by cellular injury including DNA damage (Mayer and Grummt, 2005; Kruhlak et al., 2007; Kalita et al., 2008; Pietrzak et al., 2011b). Finally, insufficiency of nucleolar transcription to replenish neuronal ribosomes has been proposed as a mechanism of neurodegeneration (Hetman & Pietrzak, accepted, TINS).

While searching for DNA damaging agents that inhibit neuronal Pol1, we observed its unexpected stimulation in response to DNA topoisomerase-II inhibitors that

was ATM dependent. Therefore, we conducted further experiments to determine whether (i) regulation of Pol1 by stimuli promoting neuronal growth required ATM, (ii) ATM was necessary for neuronal growth and (iii) ATM regulated Pol1 in immortalized proliferating cells.

Materials and Methods

Animals

All animal experiments strictly followed the protocols that were approved by the Institutional Animal Care and Use Committee of the University of Louisville and the NIH guidelines. Newborn Sprague-Dawley rats at P0 were used for cell culture. Postnatal day 7 rats were used for in vivo studies. Cerebellar and cortical $ATM^{-/-}$ mouse tissues were from a previously described line of ATM knock out mice (Herzog et al., 1998).

Materials

The following reagents were obtained from the indicated commercial sources: mouse monoclonal anti-B23 antibody and mouse monoclonal anti-ATM antibody, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); 5-ethynyl uridine (5-EU, Berry and Associates, Dexter, MI); Actin-RFP, Alexa 488-, Alexa 594-conjugated secondary antibodies, Oregon Green®-6-carboxamido-(6-azidohexanyl) triethylammonium salt, (Oregon Green®-azide), and Lipofectamine 2000, Invitrogen (Carlsbad, CA, USA); BDNF (Alomone Labs, Jerusalem, Israel); anti-ERK1/2 antibodies, anti-phospho-

(S1981)-ATM mouse monoclonal antibody, anti-phospho-(S473)-AKT rabbit polyclonal antibody, anti-phospho-(T389)-RPS6 mouse monoclonal, anti-RPS6 rabbit monoclonal, anti-MAP2 rabbit polyclonal, Cell Signaling (Beverly, MA, USA), antiphospho-ERK1/2 antibody (anti-ACTIVE_ MAPK pAb), Promega (Madison, WI, USA); horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies and LY294002, Calbiochem (San Diego, CA, USA). KU55933, SR-95531, and KU0063794, Tocris (Ballwin, MO, USA). All other reagents were purchased from Sigma (St. Louis, MO). Respective IgG antibodies were used in place of primary antibodies as a negative control and resulted in background staining (data not shown).

Cell culture

Cortical or hippocampal neurons were prepared from newborn Sprague-Dawley rats at P0 as previously described (Habas et al., 2006). Briefly, the culture medium was Basal Medium Eagle (BME) supplemented with 10% heat-inactivated bovine calf serum (Hyclone, Logan, UT), 35 mM glucose, 1 mM L-glutamine, 100 U/mL of penicillin and 0.1 mg/mL streptomycin. Cytosine arabinoside (2.5 μM) was added to cultures on the second day after seeding (day in vitro 2, DIV2) to inhibit the proliferation of non-neuronal cells. Cells were used for experiments at DIV 5-6, with the exception of neuronal activity-dependent experiments and morphogenesis experiments. Neuronal activity experiments were conducted on DIV 8 to allow for neurons to develop a network of processes with synaptic contacts and express functional NMDA-type and AMPA glutamate receptors (Papadia et al., 2005). For morphogenesis experiments neurons were transfected on DIV 3, treated on DIV 4 and fixed 24 h later (DIV5).

Serum starvation of cultured cortical neurons. Serum starvation of cultured cortical neurons has been described previously (Kharebava et al., 2008). Briefly, on DIV 5-6, cells were washed twice with serum free BME and placed in BME medium containing MK-801and AraC. Two hours later, BDNF (10ng/mL) and/or inhibitors were added to the medium for an additional 2 h.

Serum stimulation of cell lines. Immortalized cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and plated at a density of approximately 2 X 10^6 / 35 mm dish. Serum starvation was conducted by washing cells twice with serum free DMEM followed by placement in serum free DMEM supplemented with antibiotics for 12-14 h. Then, cells were placed back in the full growth medium in the presence or absence of KU55933.

Intracerebroventricular injection

ICV injection into the P7 rat has been described previously (Pietrzak et al., 2011b). Briefly, injections of 5 μL artificial cerebrospinal fluid (aCSF: 126 mM NaCl, 25 mM $NaHCO_3$, 25 mM Glucose, 1.25 mM NaH_2PO_4, 2.5 mM KCl, 1 mM $MgCl_2$) / 20% DMSO ± pharmacological agent (etoposide, ICRF-193, KU55933) were made with a Hamilton needle inserted into the right lateral ventricle at the following coordinates: 1.5 mm rostral- and 1.5 mm lateral to lambda, 2 mm deep from the skull surface. Lambda was readily identified by the underlying venous sinuses which were visualized by shining light from two focal sources placed on either side of the head, or one light source beneath the chin. After injection, animals were placed back with their mother.

Transfection of DNA

An expression plasmid for alpha-acti Red Fluorescent Protein (alpha-actin RFP) was transiently transfected into cortical neurons on DIV 3. First, the conditioned medium was removed and saved. Cells were then placed in serum-free basal Eagle's medium (Sigma) supplemented with 1.5 μL/mL 20mM glucose containing 0.5 μg of DNA mixed with 1.5 μL of Lipofectamine/well. After a 40 min incubation at 5% CO_2 and 37 °C, the transfection medium was replaced with conditioned medium. Cells were fixed for 15-30 minutes at room temperature and immunostained 2 days after transfection.

Image acquisition/Morphogenesis

Images were captured using the Zeiss AxioObserver inverted microscopes with MetaMorph or AxioVision software, respectively. Fluorescent exposure rates were kept consistent across all conditions by fluorescent filter. Images were exported as TIF files with and without scale bar annotations. Both the annotated and unannotated TIF images were further scaled in Photoshop7 (Adobe Systems Incorporated, San Jose, CA, USA) and a line of appropriate font was applied to the unannotated TIF that matched the size of the annotated TIF in Adobe Illustrator 10 (Adobe).

Morphogenesis images were captured on Zeiss Axio Observer inverted microscope and AxioVision software at 20X magnification. NeuronStudio (Wearne et al., 2005; Rodriguez et al., 2008) was used to hand-trace neurites from the soma. An average of 13 representative cell images were analyzed per condition per culture. The researcher collecting images and conducting the tracing was blinded to each of the experimental conditions while conducting this work. Images were accessed by the

presence of healthy nuclei, evaluated by Hoechst 33258 nuclear staining, alpha-actinRFP-positive fluorescence, and the absence of neurite beading (an indicator of progressing degeneration).

Drug treatment

Etoposide, ICRF-193, LY294002, U0126, KU55933, KU0063794, MK-801, SR95531 (Gabazine), and 4-AP were dissolved in dimethyl sulfoxide (DMSO). Inhibitors were added to the medium 20 minutes prior to stimulation by etoposide, ICRF-193, BDNF or Gaba/4-AP. The final media concentration of the solvents was 0.2%.

In situ run-on assay

The RNA precursor, 5-EU, was used as described previously (Gomes et al., 2011). Briefly, to label nascent RNA, glass coverslip-cultured cells were incubated with 1 mM 5-EU for one hour (37°C, 5% CO_2) followed by fixation with 4% paraformaldehyde. Co-immunofluorescence for the nucleolar marker B23 was performed according to standard protocols. Following incubations with the secondary antibodies, the 5-EU-labeled nascent RNA was detected using the previously described "click" chemistry methodology (Jao and Salic, 2008). The "click" buffer (1 M tris-HCl, pH 8.5; 100 mM $CuSO_4$, 0.5 M ascorbic acid, 5 mM Oregon Green® azide in DEPC-treated water) was applied for 30 min followed by a wash with PBS and mounting of the coverslips on to slides. The integrated brightness density of 5-EU was calculated in the

B23-positive nucleoli using the NIH freeware Image-J. For each cell, nucleolar signal was normalized against the 5-EU staining in the whole nucleus.

Western blot analysis

Western blot analyses were performed using standard protocols and illuminated with Luminol. Primary antibodies were used at 1:1000 and horseradish peroxidase-conjugated mouse or rabbit secondary was used at 1:10,000 (Hetman et al., 2002).

Immunofluorescence

Deeply anaesthetized rats were transcardially perfused with ice cold PBS followed by 4% paraformaldehyde in PBS. Following 24 h post-fixation, brains were cryoprotected for up to 72 h in 30% sucrose and sectioned on a cryostat at 25 μm sections, and mounted on slides. Sections were membrane-permeabilized using NP-40 0.5% in PBS. Sections were then incubated in blocking solution (3% bovine serum albumin, 10% normal goat serum in PBS/0.1% Triton-X100 (PBST), 1 h) followed by primary and secondary antibodies (anti-ATM, 1:100, overnight at 4°C and anti-mouse-IgG-Alexa-594 or Alexa-488, 1:100, 1 h at room temperature, respectively). Immunocytochemistry for cortical neurons was conducted similarly and concentrations as follows: anti-ATM, 1:1000, anti-mouse IgG-Alexa-594 or 488, 1:1000. Nuclei were visualized by Hoescht-33258. Then, sections were covered with coverslips that were mounted using Fluoromount G (Southern Biotech, Birmingham, AL, USA).

RNA isolation and quantitative Real-Time PCR

Following CO_2 euthanasia, the entire rat neocortex was harvested and immediately frozen on dry ice. Tissue RNA extraction was done following homogenization with a pestle grinder (Fisherbrand; Fisher Scientific, Pittsburgh, PA, USA) in 1 mL TRI Reagent (Sigma) at 4°C. Cultured cell RNA was isolated from 5×10^6 cells/sample using TRI Reagent. The random hexamers-primed cDNA synthesis, quantitative RT-PCR, and the ΔΔct-based analysis of 45S pre-rRNA/18S rRNA levels were done as previously described (Pietrzak et al., 2011b).

Oligonucleotides

Primers for rat 45S pre-rRNA and 18S rRNA have been described previously (Pietrzak et al., 2011b). Rat and mouse 18S rRNA share sequence homology. Primers for mouse pre-rRNA were as follows:

45S forward: 5'-CTC ACC CCC GGT TTG TCC T-3'; reverse: 5'-CTA CCT GGC AGG ATC AAC CA-3'

18S forward: 5'-TCA ACT TTC GAT GGT AGT CGC CGT-3'; reverse: 5'-TTC TTG GAT GTG GTA GCC GTT TCT-3'

Primers for human pre-rRNA and 18S rRNA were as follows:

45S forward: 5'-GTC AGC CCT CGA CAC AAG G-3'; reverse: 5'-GGG AGG AAG ACG AAC GGA AG-3'

18S forward: 5'-TCA ACT TTC GAT GGT AGT CGC CGT-3'; reverse: 5'-TTC TTG GAT GTG GTA GCC GTT TCT-3'

Results

DNA topoisomerase-II inhibitors induce nucleolar transcription through the activation of ATM

Etoposide is a widely-used anti-cancer drug that inhibits DNA-topoisomerase-2 (Topo2) and thereby induces DSBs, SSBs and Topo2-DNA cross-links (Nitiss, 2009a). Etoposide has previously been shown to suppress Pol1-mediated transcription at high concentrations in mouse embryonic fibroblasts (MEFs) (Kruhlak et al., 2007). Similarly, etoposide induces nucleolar stress in neurons at concentrations exceeding 10 μM for 8 h (Pietrzak et al., 2011b). While evaluating effects of lower concentrations of etoposide we were surprised to observe stimulation of Pol1. Thus, levels of pre-rRNA increased in cortical neurons exposed to 0.3 and 1 μM etoposide for 4 h (Fig. 9A). This finding led us to test another Topo2 inhibitor, ICRF-193, whose potential to induce DNA strand breaks is lower than that of etoposide (Hajji et al., 2003; Park and Avraham, 2006; Robinson et al., 2007; Nitiss, 2009b). Increased pre-rRNA levels were observed in cortical neurons that were exposed to 25 μM ICRF-193 for 4 h (Fig. 9B). We observed a similar induction of Pol1-mediated transcription in the ipsilateral cortices of neonate rats at 4 h after low dose injections of etoposide or ICRF-193 into a lateral ventricle (Fig. 9C, D).

Figure 9

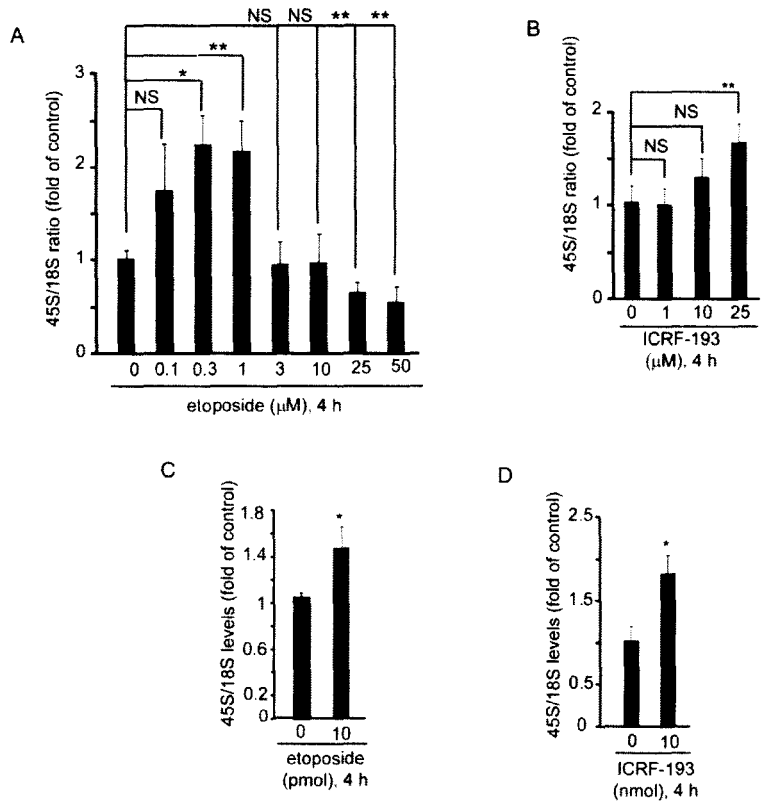

Figure 9: Topoisomerase-II inhibitors increase neuronal nucleolar transcription. ***A-B***, Cultured cortical neurons were treated as indicated for 4 h. (A) Low concentrations of etoposide increased the 45S pre-rRNA/18S rRNA ratio. Nucleolar transcription was suppressed at concentrations above 10 μM. (B) Increased levels of 45S pre-rRNA after treatment with 25 μM ICRF-193. ***C-D***, P7 rat pups (3 animals/condition) received injections of either etoposide (C), or ICRF-193 (D) into the right lateral ventricle; after 4 h, ipsilateral cortices were dissected and analyzed. In all graphs, error bars indicate SEM; *, $p<0.05$; **, $p<0.01$; in A-B, three independent cultures were analyzed.

As in non-neuronal systems, both etoposide and ICRF-193 activate DNA damage signaling including ATM. We wondered whether activation of Pol1 in neurons treated with these drugs is regulated by ATM. First, we confirmed that 1 and 10 μM etoposide (Fig.10A), as well as 10 and 25 μM ICRF-193 (Fig. 10B), increased ATM autophosphorylation at S1981 suggesting activation of this kinase. Importantly, concentrations that caused ATM activation overlapped with those activating Pol1. We then used the ATM-specific inhibitor (Hickson et al., 2004; Li and Yang, 2010), KU55933 (K5), to test the Pol1 effects of Top2 inhibitors upon blocking ATM. At 10 μM, K5 suppressed the etoposide-induced autophosphorylation of ATM at S1981 (Fig. 10C). It also suppressed Pol1 activation either by etoposide (1 μM) (Fig. 10D) or ICRF-193 (25 μM) (Fig. 10E). Finally, the etoposide-mediated induction of Pol1-driven transcription in the nucleolus and its prevention by the ATM inhibitor were confirmed using *in situ* run-on assay with the RNA precursor, 5-ethynyluridine (5-EU) followed by immunostaining for the nucleolar marker B23 (Fig. 10F,G). These results also confirmed that at low concentrations of etoposide which activated Pol1, nucleolar integrity was not affected. Together, these observations suggest that in Topo2-inhibited neurons ATM activation stimulates Pol1-driven transcription.

Figure 10

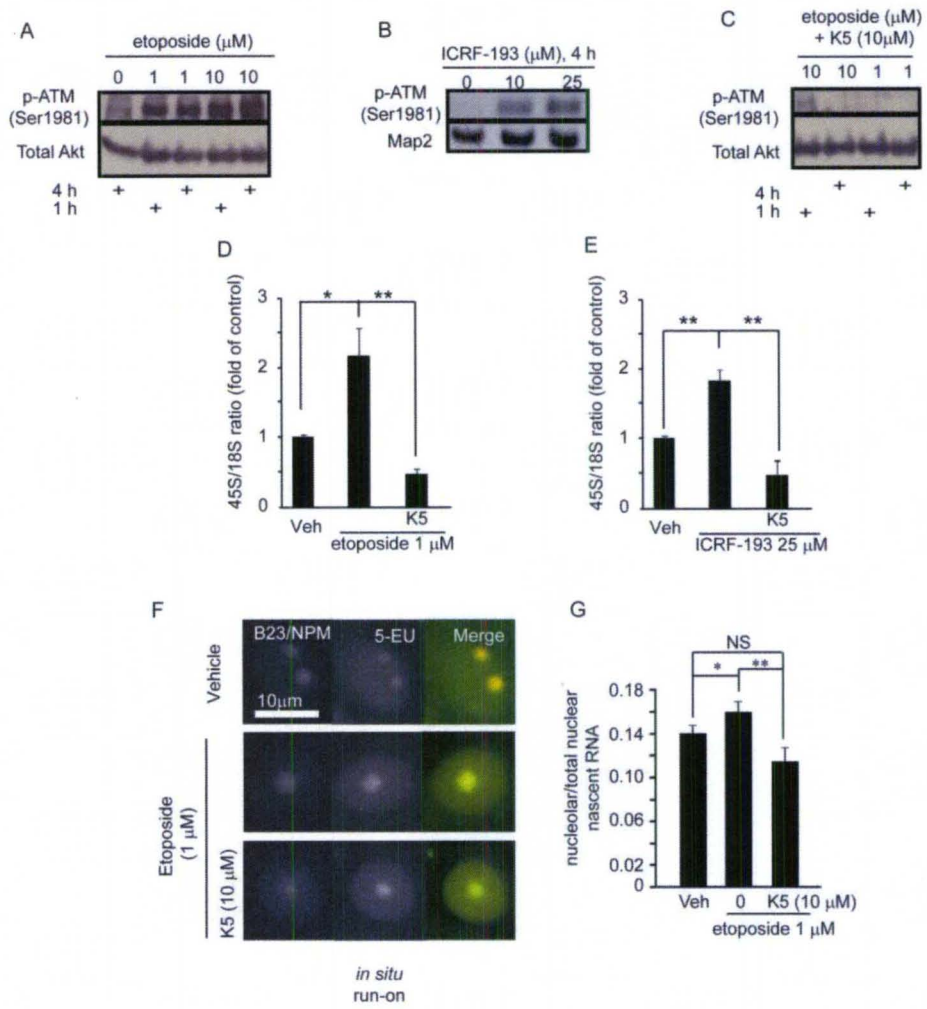

Figure 10: Pol1-mediated transcription of cultured cortical neurons increases in response to ATM activation by Topo2 inhibitors.

A-B, Etoposide and ICRF-193 activated ATM as indicated by its increased autophosphorylation at the S1981 residue. ***C***, The specific ATM inhibitor, KU55933 (K5), attenuated the etoposide-mediated activation of ATM. (A-C) representative Western blots are shown. Equal protein content on the blots was ensured by reprobing them for either total MAP2, or running a new gel with the equal protein concentrations and probing for AKT. ***D-E***, ATM inhibition with 10 μM K5 attenuated the effects of Topo2 blockers on neuronal nucleolar transcription. Averages of 3 independent experiments are shown. ***F-G***, Etoposide-mediated accumulation of nascent RNA in the nucleolus is prevented by K5. Increased nascent RNA levels in neuronal nuclei were observed after etoposide treatment, and were reduced following ATM inhibition.

Cortical neurons were treated with vehicle, 1 μM etoposide, or co-treated with etoposide 1 μM and 10 μM K5 for 3 h followed by 1 h incubation with the RNA precursor 5-ethynyluridine (5-EU, 1 mM). After fixation and co-immunofluorescence for the nucleolar marker B23, nascent RNA was visualized using "click" chemistry (see methods for more details). Etoposide increased the ratio between nucleolar- and nuclear- nascent RNA levels, whereas K5 abolished that effect. For nascent RNA quantifications, the nucleolar compartment was defined by B23 immunofluorescence. (G) Data are averages of at least 40 randomly selected individual neurons/condition from 3 independent experiments. In all graphs, error bars indicate SEM; *, $p<0.05$; **, $p<0.01$.

The role of ATM in maintaining neuronal Pol1 activity under basal conditions

To further evaluate the involvement of ATM in regulating nucleolar transcription in neurons, we first characterized effects of its pharmacological inhibitor K5 on other signaling pathways that were previously implicated in activation of Pol1 in response to growth factors. We treated dissociated cortical neurons for 4 h with K5 as well as inhibitors of ERK1/2- (U0126), PI3K- (LY29004, (LY)), and mTOR- (KU0063794, (K006)) pathways. We then evaluated the activation status of ERK1/2, and mTORc1 by monitoring phosphorylations of ERK1/2 and RPS6 (T183/Y185, S235/236, respectively). In addition, phosphorylation of AKT at S473 was studied as it may be carried out by ATM itself or by DNA-PK or mTORc2 (Fig. 11A). Basal ATM inhibition did not affect ERK1/2, and decreased the phosphorylation of AKT, and RPS6 compared to vehicle. These results support the notion that ATM contributes to signaling networks that mediate growth factor responses. Consistent with that, ATM inhibition reduced basal pre-rRNA levels to a similar extent as did inhibition of ERK1/2, PI3K, and mTOR (Fig. 11B). In addition, intracerebroventricular injection of K5 reduced basal pre-rRNA levels in the ipsilateral cortex of neonate rats (Fig. 11C). To identify whether ATM inhibition affected global transcription, we again employed the *in situ* run-on assay to label *de novo* transcripts following 4 h treatment with K5 (10 μM). Decreased content of nascent RNA

was observed in neuronal nucleoli in response to ATM inhibition (Fig. 11D). The decreased ratio between nucleolar- and total nuclear nascent RNA levels suggests the anti-transcriptional effects of K5 were relatively greater in the nucleolus (Fig. 11E). In concert with prior observations that ATM is concentrated in the nucleoli of human neurons (Gorodetsky et al., 2007), ATM immunofluorescence co-localized with the nucleolar sites of nascent RNA accumulation (Fig. 11F). Finally, lower pre-rRNA levels were also observed in the cerebella of 5 week old $ATM^{-/-}$ mice (Fig. 11G). Together, these findings indicate that ATM regulates Pol1, not only upon challenge with DNA double strand breaks, but also under basal conditions. Such effects may be related to ATM's contribution to the signaling networks in control of cell growth.

Figure 11

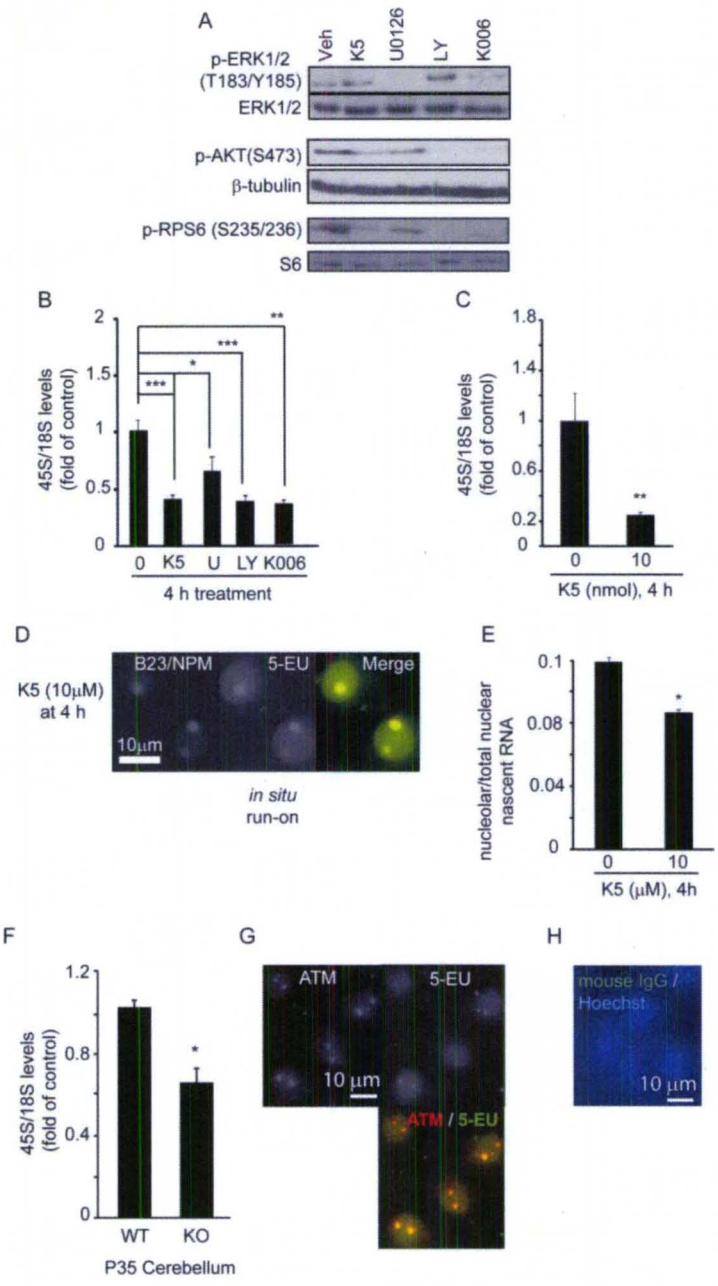

Figure 11: ATM regulates neuronal Pol1 under basal conditions
A, Cortical neurons were treated with K5 as well as inhibitors of several well established signaling pathways that control neuronal growth and mediate effects of growth factors on ribosomal biogenesis, including ERK1/2, (U0126 at 50 μM); AKT (LY294002 at 30 μM); mTOR, (KU 0063794, (K006) at 0.3 μM)), and K5 (10 μM). Their effects on

phosphorylation of ERK1/2, AKT, and RPS6 were revealed by Western blotting (p-T183/Y185, ERK1/2; p-S473, AKT; p-S235/236, RPS6). While K5 did not affect ERK1/2 activity, it did reduce phosphorylations of AKT and RPS6 phosphorylation suggesting that at least in developing neurons ATM plays a role in growth factor signaling. ***B***, Cortical neurons were treated with inhibitors as in A. All drugs lowered pre-rRNA levels. Averages of 3 independent experiments are shown. ***C***, P7 rat pups (3 animals/condition) received injections of K5 (10 nmoles) into the right lateral ventricle; after 4 h, pre-rRNA levels in the ipsilateral cortex declined. ***D-E***, Decreased nascent RNA levels in neuronal nuclei after K5 treatment. (D) Cortical neurons were treated with K5 (10μM) or vehicle followed by incubation with the RNA precursor 5-ethynyluridine (5-EU, 1 mM) for 1 h. After fixation and co-immunofluorescence for the nucleolar marker B23, nascent RNA was visualized using "click" chemistry. (E) K5 decreased the ratio between nucleolar- and nuclear nascent RNA levels. Data are averages of at least 40 randomly selected individual neurons/condition from 3 independent experiments. ***F***, ATM colocalizes with 5-EU in the nucleolus. ***G***, Decreased pre-rRNA levels in the cerebellum of 5-week old $ATM^{-/-}$ mice. ***H***, Representative mouse IgG control immunostaining showing only Hoescht-33258 (blue). In all graphs the averages of 3 independent experiments are shown, error bars indicate SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

ATM's contributions to the activation of neuronal Pol1 by BDNF and increased synaptic activity

BDNF provides an extracellular signal that stimulates neuronal growth by increasing Pol1 activity (Gomes et al., 2011). Therefore, we wondered whether ATM played a role in BDNF-mediated activation of Pol1. In cultured cortical neurons treated with 10 ng/ml BDNF for 4 h, the activation of growth promoting signaling pathways including ERK1/2, AKT and mTORc1 was indicated by increased phosphorylation levels of T183/Y185, S473 and S235/236, respectively. Activation of ERK1/2 was unaffected by K5. Conversely, K5 prevented AKT activation and reduced mTORc1 activity towards RPS6. However, K5 interference with mTORc1 activation was weaker than that of the

PI3K inhibitor, LY, or the mTOR inhibitor, KU006 (Fig. 12A). These results suggest a contribution by ATM to the growth promoting signaling network activated by BDNF.

To determine effects of K5 on BDNF-induced activation of Pol1, two BDNF stimulation conditions were tested. These paradigms included 2 h BDNF treatment in full growth medium or 2 h BDNF treatment in trophic deprivation medium, which was added to cells 2 h before the BDNF addition. The latter paradigm was used to clearly separate K5 effects on the BDNF response from those of trophic factors that are present in full growth medium. Indeed, such factors may employ ATM to stimulate Pol1 as K5 lowered pre-rRNA levels in full media, but not in trophic deprived neurons (Fig. 12B, C). However, BDNF induced nucleolar transcription under both stimulation conditions, while K5 reduced the BDNF responses (Fig 12B, C). Similar reductions were also observed under inhibition of PI3K and mTOR. Finally, K5 reduced elevated accumulation of nascent RNA in the nucleoli of BDNF-stimulated neurons (Fig. 12D). Taken together, these data suggest that in addition to those PI3K family members that are well established regulators of neuronal growth, ATM is critical for the activation of Pol1 in a growth-driving response to BDNF.

Figure 12

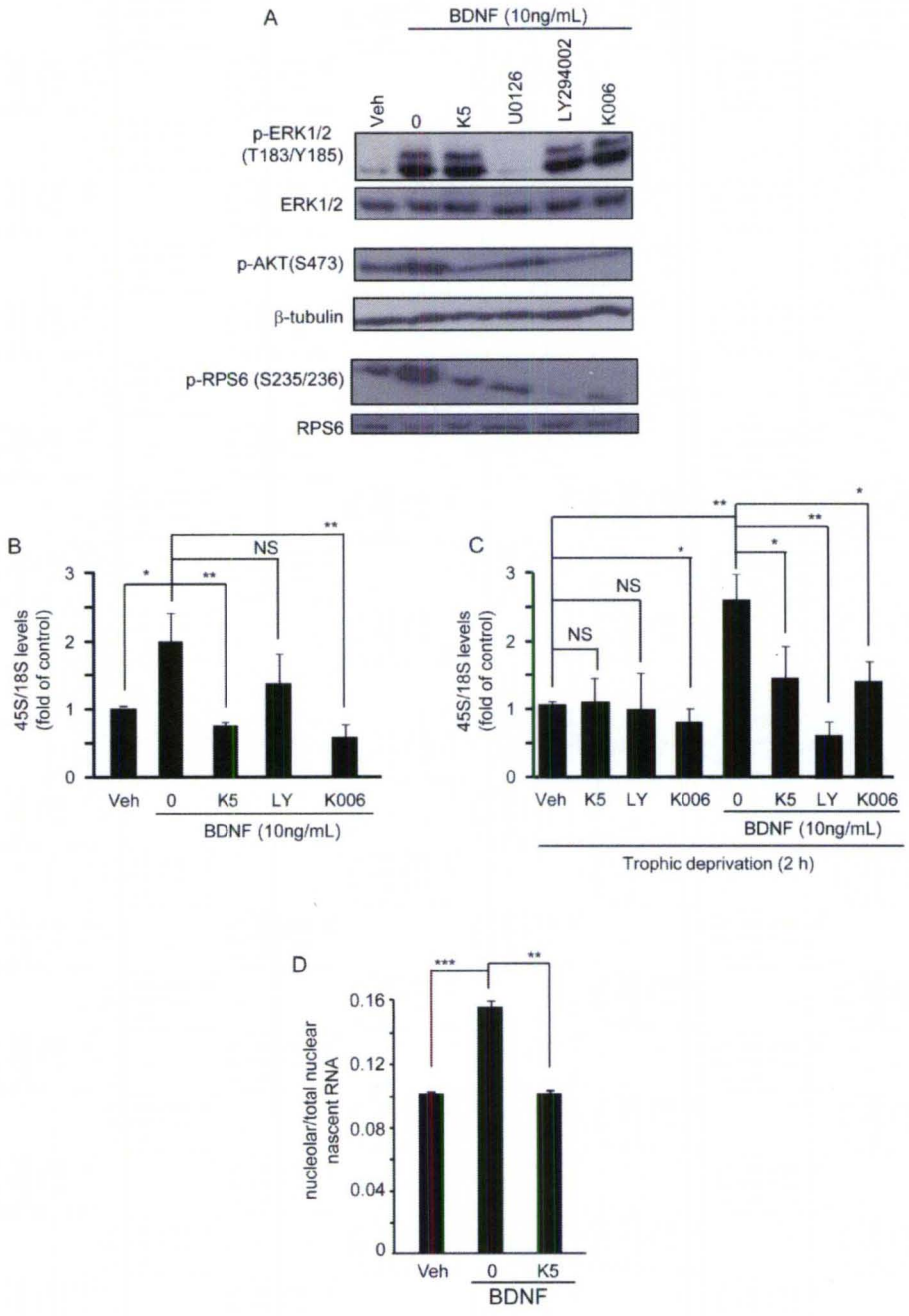
A
BDNF (10ng/mL)
Veh
0
K5
U0126
LY294002
K006
p-ERK1/2 (T183/Y185)
ERK1/2
p-AKT(S473)
β-tubulin
p-RPS6 (S235/236)
RPS6
B
45S/18S levels (fold of control)
NS
Veh
0
K5
LY
K006
BDNF (10ng/mL)
C
NS
Trophic deprivation (2 h)
D
nucleolar/total nuclear nascent RNA
BDNF

Figure 12: Activation of Pol1 in BDNF-stimulated neurons is dependent on ATM, PI3K and mTOR.
A, Treatment of cortical neurons with BDNF (10ng/mL) for 4 h increased phosphorylations of ERK1/2 (T183/Y185), AKT (S473) and RPS6 (S235/236) suggesting activation of ERK1/2, PI3K/AKT and PI3K/mTORc1, respectively. ERK1/2 activation was not attenuated by inhibiting ATM (K5), PI3K (LY) or mTOR (K006). However each of these inhibitors reduced AKT- and mTORc1 activation. Although K5 prevented AKT activation, its inhibitory effects on BDNF-mediated activation of mTORc1 were smaller as compared to LY or KU006. ***B-C***, BDNF activation of Pol1 was ATM-dependent. Cortical neurons were treated with BDNF for 2 h as indicated. Inhibition of ATM, PI3K or mTOR prevented BDNF-induced increases of pre-rRNA levels. Data are from three independent experiments. ***D***, Co-treatment of BDNF and K5 decreased nascent RNA levels in neuronal nuclei. Cortical neurons were treated with vehicle or with BDNF in the presence and absence of K5 (10 μM) followed by incubation with the RNA precursor 5-ethynyluridine (5-EU, 1 mM) for 1 hr, as in Figure 11. Quantifications are averages of at least 40 randomly selected individual neurons/condition from 3 independent experiments. In all graphs, error bars indicate SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

As synaptic activity also stimulates neuronal growth, we examined whether it regulates Pol1 through the ATM pathway. To stimulate synaptic activity, cultured cortical neurons were treated for 4 h with SR95531 (Gabazine), a γ-aminobutyric $acid_A$ ($GABA_A$) receptor antagonist, and 4-aminopyridine, a selective potassium channel blocker (Kv1). Confirming prior reports that such treatment stimulates synaptic activity and increases ERK1/2 signaling (Ghosh and Greenberg, 1995; Krapivinsky et al., 2003), we observed increased phosphorylation of ERK1/2 that was unaffected by K5 (Fig. 13A). Therefore, K5 did not interfere with the neuroexcitatory effects of Gabazine/4AP. However, it did block the Gabazine/4AP-mediated induction of Pol1 (Fig. 13B). This inhibitory effect was similar to that of the ERK1/2 pathway inhibitor, U0126, and the electrical activity inhibitor, tetrodotoxin (TTX), and the NMDA receptor antagonist, MK801. These data suggest that enhanced synaptic activity stimulates Pol1-driven

transcription in an ATM-dependent manner. Hence, ATM is a critical transducer for the growth-associated activation of nucleolar transcription in response to such growth promoting stimuli as BDNF and synaptic activity.

Figure 13

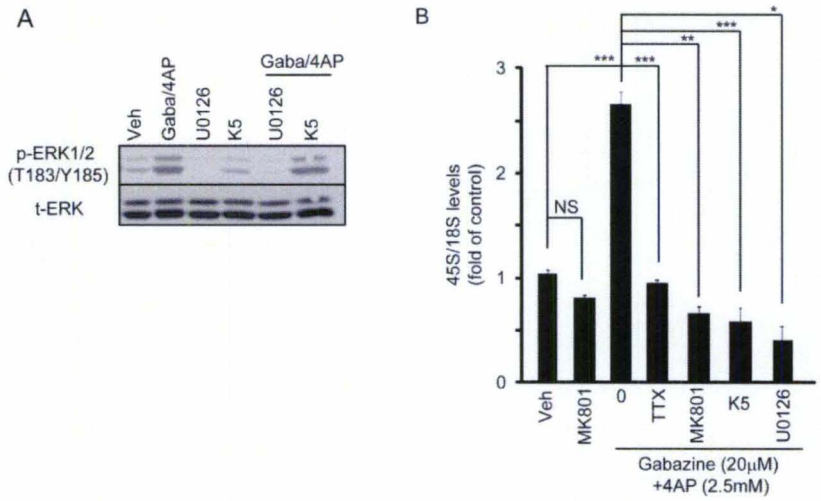

Figure 13: Enhanced synaptic activity potentiates neuronal nucleolar transcription via ERK1/2 and ATM.
A, DIV 8 cortical neurons were treated with the $GABA_A$ receptor antagonist, Gabazine (Gaba) and voltage-gated potassium channel blocker, 4-aminopyridine (4-AP) for 4 h. This treatment activated ERK1/2. ***B***, Enhanced synaptic activity drove neuronal nucleolar transcription through an ERK1/2 and ATM dependent pathway. DIV 8 cortical neurons were treated with inhibitors as indicated. 20 minutes later they were treated with Gaba and 4-AP or vehicle for 4 h. As anticipated, the sodium channel blocker, tetrodotoxin (TTX), and MK801 attenuated the neuronal nucleolar transcription induction following Gaba/4-AP treatment. Similarly, ERK1/2 inhibition and ATM inhibition in the presence of Gaba/4-AP also attenuated the transcriptional induction. In all graphs the averages of 3 independent experiments are shown, error bars indicate SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Inhibition of ATM reduces neuronal growth

To test whether ATM is required for neuronal growth we transfected cortical neurons with an expression vector for RFP to label cell bodies and neurites of a few cells (14A). After 24 h, pharmacological inhibitors of ATM or mTOR were added for the next 24 h. To exclude possible interference of negative survival effects of such inhibitors with their potential anti-growth actions, a fraction of the transfected (i.e. RFP-positive) cells with "healthy," non-condensed morphology of nuclear chromatin were determined following nuclear counterstaining with Hoechst33258. None of the inhibitors significantly affected neuronal survival (Fig. 14B). Conversely, total neurite length decreased in both ATM- and mTOR-inhibited neurons (Fig. 14C). Although the neuritic branching was unchanged, as was the number of primary dendrites (Fig. 14D-E), the Sholl analysis indicated decreased neurite complexity following ATM or mTOR inhibition (Fig. 14F). In neurons that were stimulated with BDNF, the growth response to this neurotrophin was also sensitive to ATM or mTOR inhibitor (Fig. 15). Under BDNF-stimulated conditions, the K5 inhibitor did not affect neuronal survival (Fig. 15B). However, it significantly attenuated BDNF-mediated increases in dendrite total length and the number of primary dendrites (Fig. 15C). In addition, BDNF-mediated increases in dendritic length, branching and overall complexity were attenuated by ATM inhibition as revealed by the Scholl analysis (Fig 15F). Collectively, these results suggest that in addition to mTOR, ATM is a major regulator of neuronal growth. Such a notion is consistent with a critical role of either pathway in driving neuronal production of ribosomes.

Figure 14

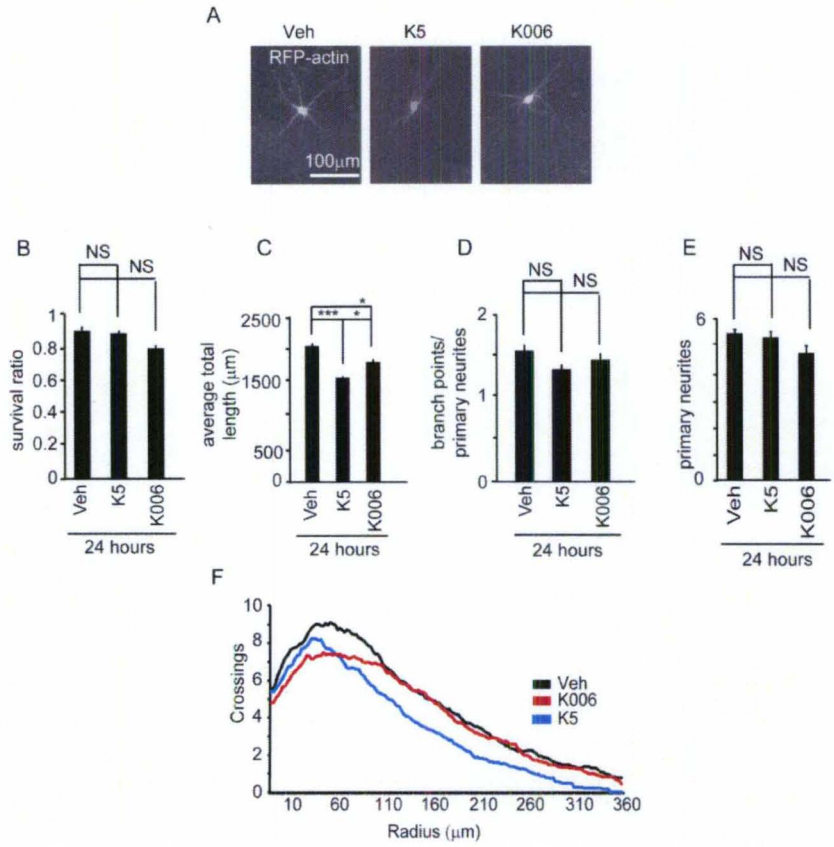

Figure 14: Pharmacological inhibitor of ATM reduced neurite length and neurite complexity under basal conditions.

Cortical neurons were transfected on DIV 3 with an RFP expression vector (0.25 μg plasmid DNA/5 X 10^5 cells). Treatments with a vehicle (Veh, 0.2%DMSO), 10 μM K5 or 0.3 μM K006 were initiated on DIV4 and continued for 24 h. ***A***, Representative photomicrographs of individual transfected neurons following the inhibitor treatment. ***B-F***, Morphometric analysis of ATM- or mTOR-inhibited neurons. ATM or mTOR inhibition for 24 h had no significant effects on cell survival as determined by the fraction of RFP-positive neurons with a "healthy," non-condensed chromatin that was visualized by counterstaining with Hoechst33258. Both inhibitors reduced total neurite length (C) without affecting neuritic branching (D) or the number of primary dendrites (E). However, Scholl analysis indicated reduced neuritic complexity following treatment with either inhibitor. The effect of the ATM inhibitor was mainly on the distal neurites while the mTOR inhibitor affected more proximal processes (F). (B-F), data are from a minimum of 36 cells per condition that were analyzed in three independent experiments, error bars indicate SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Figure 15

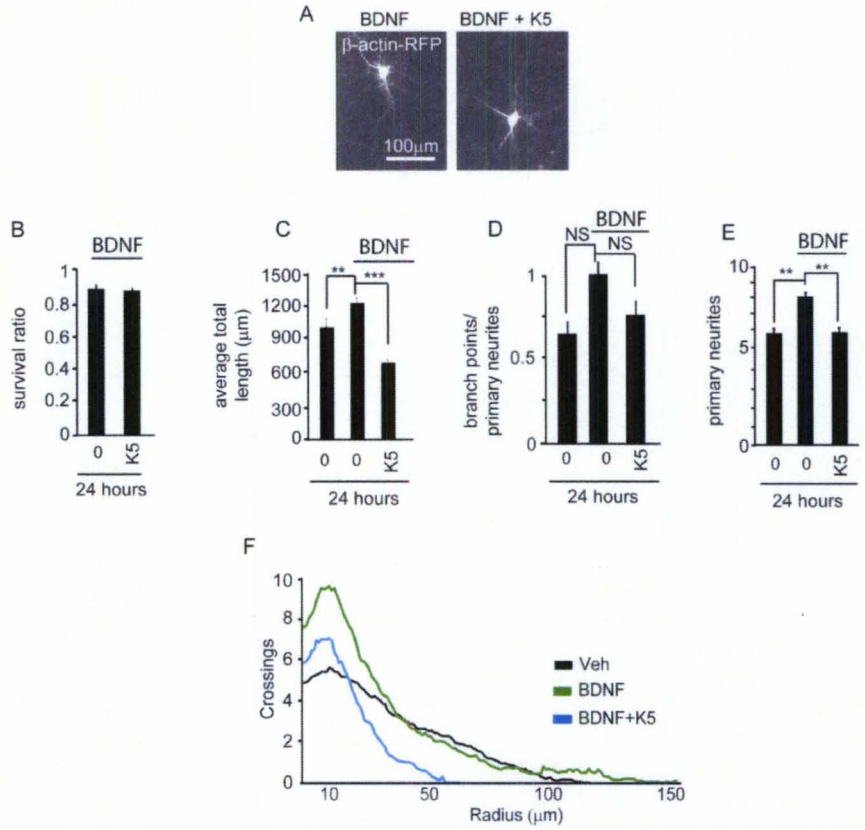

Figure 15: Pharmacological inhibitor of ATM reduced neurite length and neurite complexity in response to BDNF-stimulated growth

Cortical neurons were transfected on DIV 3 with an RFP expression vector (0.25 μg plasmid DNA/5 X 10^5 cells). Treatments with vehicle (Veh, 0.2% DMSO), BDNF (20 ng/mL), and BDNF plus inhibitor: 10 μM K5 treated on DIV4 and continued for 24 h. ***A***, Representative photomicrographs of individual transfected neurons following the indicated pharmacological treatment. ***B-F***, Morphometric analysis of BDNF-stimulated ATM- or mTOR-inhibited neurons. (B) ATM inhibition in the presence of BDNF for 24 h had no significant effects on cell survival as determined by the fraction of RFP-positive neurons with a "healthy," non-condensed chromatin that was visualized by counterstaining with Hoechst33258. ATM inhibition reduced total dendritic length (C), did not significantly affect neuritic branching (D), but did attenuate the number of primary dendrites (E). Scholl analysis indicated reduced neuritic complexity following K5 treatment (F). In B-F, data are from a mean of 36 cells per condition that were analyzed in three independent experiments, error bars indicate SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

ATM-mediated regulation of Pol1 in dividing cells

To test the possibility that ATM regulates Pol1 activity in proliferating cells, we performed serum stimulations of the human neuroblastoma SH-SY5Y cell line and human cervical cancer HeLa cell line with or without the ATM inhibitor. Under such conditions, serum mediated induction of pre-rRNA expression was abolished in SH-SY5Y cells (Fig. 16A). In HeLa cells K5 reduced the serum-mediated increase in pre-rRNA levels by 59% (Fig. 16C). These observations suggest that ATM is required for ribosomal biogenesis not only in rodent neurons but also in dividing human cancer cell lines.

Figure 16

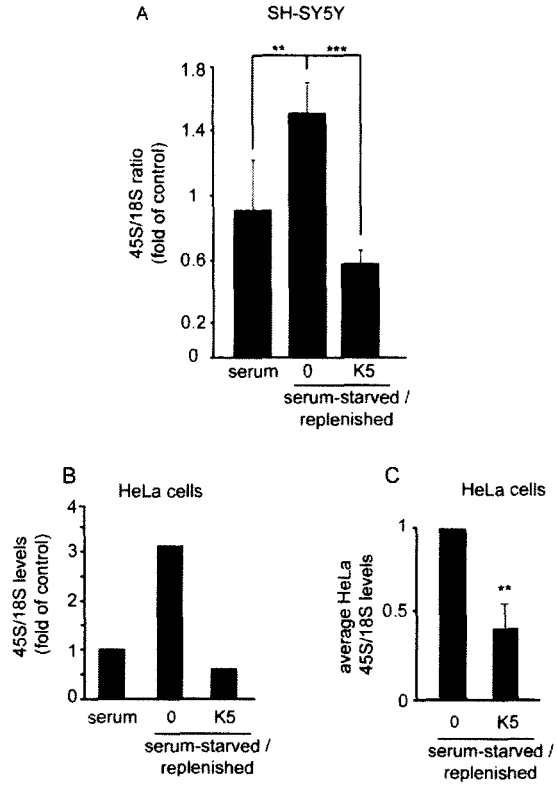

Figure 16: Requirement of ATM for serum-mediated activation of Pol1 in dividing human cell lines.

A-C, After overnight serum deprivation of human SH-SY5Y neuroblastoma cells (SH-5Y) or HeLa cervical cancer cells (HeLa), restoration of the full growth medium that contained 10% FBS activated Pol1 as suggested by increased pre-rRNA levels at 4 h after starting such stimulation. Pol1 activation was reduced in the presence of the ATM inhibitor K5 (10 μM). (A) The average of 3 independent experiments are shown. (B) Data from a representative experiment; (C) the mean percent of the serum-mediated increase in pre-rRNA levels from 4 independent HeLa cell experiments is depicted. In all graphs, error bars indicate SEM; **, $p<0.01$; ***, $p<0.001$.

Discussion

We report that Pol1-driven transcription, the rate-limiting step of ribosomal biogenesis, is regulated by ATM in both postmitotic neurons and in dividing cells. In primary neurons exposed to low level DNA damage, BDNF or enhanced synaptic activity, ATM was necessary for increases in Pol1 activity. In human cell lines of tumor origin, ATM was essential for Pol1 activation by serum. In addition, ATM inhibition reduced BDNF-stimulated neurite outgrowth. Collectively, ATM appears to be a major regulator of neuronal growth with at least one of its growth promoting mechanisms being stimulation of nucleolar transcription.

The effects of ATM inhibition on ribosomal biogenesis and neuronal growth are similar to those affected by interference with the ERK1/2- or the mTOR signaling pathways. In cultured neurons, ATM inhibition reduced activity of the PI3K/AKT and the PI3K/mTOR pathways without affecting ERK1/2. As PI3K/mTOR signaling regulates Pol1 and is essential for neuronal growth, pro-ribosomal and pro-growth effects of ATM may be due to its role in activation of mTOR. Indeed, ATM has been implicated as a growth factor-stimulated kinase for AKT on residue S473 which by activating AKT could regulate mTOR. Alternatively, being somewhat similar to mTOR, ATM could offer a parallel pathway that would be activated by similar upstream mechanisms and target similar downstream substrates. In support of these notions, the principal activator of mTOR, Rheb, can also interact with ATM (Long et al., 2005). Similarly, 4E-BP has been shown as a common substrate for both kinases (Yang and Kastan, 2000; Hannan et al., 2003). In neurons ATM is found in the cytoplasm, at the membrane, in neurites and

in the nucleolus. As such, it may interact with both mTOR activators and a wide spectrum of critical pro-growth substrates of that pathway. Finally, the ATM localization to the nucleolus may offer a unique position to regulate ribosomal production. While at least two nucleolar regulators of that process including nucleophosmin and nucleolin have been shown to be direct substrates of ATM (Nalabothula et al., 2010), the impact of their ATM-mediated phosphorylations on Pol1 activity remains to be determined.

We previously identified Pol1 as a major neuronal growth effector for the BDNF-activated ERK1/2 pathway (Gomes et al., 2011). Therefore, it is tempting to speculate that Pol1 may be equally important for the neuronal growth regulation by other growth promoting pathways such as mTOR or ATM. Conversely, there may be additional mechanisms by which these kinases promote neuronal growth. Such mechanisms may include regulation of neuronal cytoskeleton and stimulation of local protein synthesis. Relative contributions of ribosomal biogenesis, translational regulation and cytoskeletal dynamics to the ATM effects on neuronal growth are to be determined.

Autophosphorylation of ATM on S1981 is a well-established indicator of its activation by DNA damage or oxidative stress (Bakkenist and Kastan, 2003; So et al., 2009; Guo et al., 2010a). It was also observed during retinoic acid- (RA) induced differentiation of SH-SY5Y neuroblastoma cells (Fernandes et al., 2007). However, no evidence for S1981 autophosphorylation has been presented in a paradigm of insulin-mediated activation of ATM (Yang and Kastan, 2000). Similarly, while neuronal ATM was strongly autophosphorylated in response to Topo2 inhibition, no change in pS1981

levels could be detected after BDNF stimulation or enhancement of synaptic activity (data not shown). Therefore, growth-promoting signals may activate ATM via alternative mechanisms that do not involve its autophosphorylation at S1981. Our future studies will address these mechanisms, including the possibility that neurotrophic factor-activated small GTPases, such as Rheb, activate ATM in the absence of DNA damage.

There is growing evidence that reduced ribosomal biogenesis contributes to neurodegeneration. For instance, inducible disruption of Pol1 activity by knocking out its co-activator TIF-1A in mature mouse neurons resulted in degeneration of various neuronal populations including cortical and hippocampal neurons, striatal neurons and substantia nigra neurons. In the latter system, Pol1 inhibition also led to oxidative stress. Partial insufficiency of neuronal nucleolar transcription has been demonstrated in Alzheimer's disease and associated with increased oxidation of rRNA and ribosomal failure (Ding et al., 2005). Such effects were also observed at the early stages of AD (Ding et al., 2006). Interestingly, iron-catalyzed Fenton reactions have been proposed as a primary source of rRNA damage in AD brains (Honda et al., 2005). In addition, degeneration of striatal neurons in Huntington's disease has been associated with HD-linked mutations in huntingtin that directly block the Pol1-specific transcriptional regulator, UBF (Lee et al., 2011). Therefore, it is tempting to speculate that A-T-associated neurodegeneration, which is accompanied by oxidative stress and attenuated by iron complexing, may be at least in part due to insufficient ribosomal biogenesis. Such insufficiency could slow the rate of replacement of damaged ribosomes, reduce protein synthesis capacity, and trigger neuronal atrophy and degeneration. Our future

studies will evaluate whether nucleolar impairment contributes to neurodegeneration in A-T.

In addition to neurodegeneration, A-T patients suffer immune deficits, and demonstrate retarded growth and accelerated aging. At least some of these defects were associated with reduced proliferation of ATM-deficient cells. We found that in addition to neurons, ATM regulated Pol1-mediated transcription in proliferating cells. Intriguingly, Pol1 activity is a critical regulator of cell growth and division (Drygin et al., 2010). In fact, Pol1 is the major effector for such principle cell cycle driver as c-Myc. Unsurprisingly, in most cases of oncogenic transformation, Pol1 activity is deregulated by loss of function mutations in tumor suppressor genes, or gain of function mutations in oncogenes (Drygin et al., 2010). Interestingly, reduced proliferation and premature senescence in ATM-null mouse fibroblasts is rescued by inhibiting the nucleolar sensor protein and a negative regulator of ribosomal biogenesis, $p19^{ARF}$ (Kamijo et al., 1999). Thus, it is likely that insufficient ribosomal biogenesis may account for the growth defects of A-T cells, thereby contributing to at least some non-neurological symptoms of that disease. Finally, blocking ATM may offer yet another strategy to block tumor growth by interfering with ribosomal biogenesis.

Collectively, we demonstrate a new role of ATM in the regulation of ribosomal biogenesis and neuronal growth. While such activity goes beyond its well-established role in the DNA damage response, this function fits well with its similarities to the growth-promoting members of the PI3K family, including PI3K and mTOR. Therefore, one may consider that functional specialization of the members of this family is not

absolute. Rather, functional redundancies and/or mutual regulatory connections between PI3K family members contribute to the integration of information about genome integrity, nutrient availability and growth factor exposure.

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CHAPTER IV

DISCUSSION

Restatement of the key findings

These studies concentrated on describing the regulation of Pol1-mediated transcription in neurons. In Chapter II, I reported that etoposide treatment suppressed Pol1 transcription and disrupted nucleolar integrity in cortical and hippocampal neurons of whole rats irrespective of developmental stage. However, in the developing, but not the mature brain, etoposide induced apoptosis. Additionally, other experiments performed in the Hetman laboratory in connection with these studies have demonstrated that in etoposide-treated neurons SSBs and/or protein DNA adducts provided the main interference with Pol1, while DSBs were the main trigger for apoptosis (Pietrzak et al., 2011b). Taken together these findings suggest that the nucleolus may play a role of a genomic integrity sensor in both young and adult neurons. In addition, they suggest that the aging- or neurodegeneration-associated accumulation of SSBs or large DNA adducts may disrupt ribosomal biogenesis thus compromising protein synthesis and leading to neuronal atrophy.

In Chapter III, I report the identification of ATM as a novel regulator of Pol1-mediated transcription in postmitotic neurons and in proliferating cell lines. These findings suggest that reduced production of ribosomes may contribute to the A-T-associated neurodegeneration and impairment of cellular proliferation. Therefore, in addition to being a DNA repair disorder, A-T may also be a ribosomal disease.

Collectively, these findings suggest that Pol1-mediated transcription is a target for the neurodegeneration associated forms of DNA damage including SSB and DNA adducts and the neurodegeneration-inducing deficiencies in ATM signaling.

Limitations and alternative interpretations

In Chapter II, etoposide was injected directly into the lateral ventricle of neonate rats, while in the adult rats the delivery was via the intracarotid artery. These different methods of delivery required different doses (Maeda et al., 1999). Though I witnessed comparable effects on the activity of nucleolar transcription and nucleolar disruption in both treatments, one may question whether these two methods are comparable. Etoposide delivery via the intracarotid artery following blood brain barrier disruption has been used in the clinic (Madajewicz et al., 1991), but this method may affect other systems upon delivery. The toxicity evaluations of humans treated with etoposide via the intracarotid artery indicated only neurological effects on few patients and hematomas at injection sites (Madajewicz et al., 1991). In adult rats treated through the intracarotid artery, etoposide caused hypoactivity and slowed breathing 5 h after treatment. Conversely, neonate rats treated with etoposide directly to the lateral ventricle were nursing within 1 h after treatment and survived up to 72 h after treatment. Unfortunately, adult rat longevity post-treatment was not evaluated beyond 6 h. However, a decreased dose of 5 mg/kg delivered via the intracarotid artery allowed survival up to 72 h. Hypoactivity and slowed respiration was witnessed under this treatment condition for up to 2 h after treatment, however nucleolar disruption was not noted microscopically in either of 2 animals treated with this dose. These findings are consistent with the lack of

biological effect observed by other researchers with a dose of 3 mg/kg delivered through the intracarotid artery (Maeda et al., 1999). Conversely, the dose delivered to the neonates caused neuronal nucleolar stress and apoptosis. Data from etoposide treatment *in vitro* may offer an explanation as to the lack of nucleolar effect after 5 mg/kg of etoposide treatment *in vivo*. Doses of etoposide treatment below 25 μM were unable to cause nucleolar transcription in cultured neonate cortical neurons. Instead, treatments below this concentration caused apoptosis, while the higher doses of etoposide, which induced more SSBs and DNA-Topo2 adducts caused nucleolar stress (Pietrzak et al., 2011b). Upon intracarotid delivery some drug is likely taken up by the arterial endothelium, and is relatively diffused by its path throughout the ipsilateral hemisphere, while delivery to the ventricle concentrates the treatment to a relatively smaller area. Further, the osmotic disruption of the blood brain barrier increases drug delivery efficiency, but remains less efficient for drug delivery than ICV delivery. These elements likely support the *in vivo* absence of nucleolar stress following 5 mg/kg doses of etoposide in the adult. As the biological effect on the nucleolus was comparable between intracarotid delivery of 15 mg/kg and ICV neonate delivery of 10 nmoles, in terms of Pol1 suppression and observed cell frequency (i.e., all cells), the doses are likely comparable despite different systems of delivery.

NPM/B23 translocation to the nucleoplasm occurred in all analyzed cells following 15 mg/kg of etoposide delivery to the adult rat brain, yet apoptosis was absent, which may suggest a neuronal protective threshold, rather than absence of biological effect. Instead, the absence of apoptosis is a function of neuronal maturation. Adult neurogenesis cannot replace all neurons in the brain, thus longevity of adult neurons is

required. Therefore, with increasing age, neuronal protection from apoptosis gradually becomes the default setting, whereas prior to maturity apoptosis was the default setting. Embryonic day 15-16 mouse cortical neurons maintained in culture for 25 days have a reduced apoptotic response following etoposide treatment than do like-treated neurons cultured for 5 days. This effect is partially associated with the reduction of p53-responsiveness in 25-day cultured neurons compared to 5-day cultured neurons (Martin et al., 2009), and the nearly complete reduction of 5-day cultured apoptosis in $p53^{-/-}$ neurons compared to the remaining apoptotic response in 25-day cultured $p53^{-/-}$ neurons. These results suggest that p53-mediated apoptosis in response to DNA damage decreases with increasing neuronal age (Martin et al., 2009). Further, the tyrosine kinase c-Abl, binds p53 in response to DNA damage and suppresses apoptosis in mature neurons and its deletion is protective in immature neurons (Martin et al., 2009).

The increased threshold to apoptosis is at least partially attributed to the reduction of pro-apoptotic molecules with increasing age, however increased expression of anti-apoptotic molecules can also increase the cellular threshold to apoptosis in mature neurons (Benn and Woolf, 2004). For instance, such anti-apoptotic mediators are likely responsible for the neuronal maturation-related delay in the release of cytochrome C which is a critical step in apoptosis (Wright et al., 2007). Apaf1 binds cytochrome C in the cytosol, and together they form the apoptosome. Apaf1 expression is suppressed in mature neurons, thus altering the recruitment of caspases to the apoptosome. Mature neurons grown in culture are sensitive to etoposide treatment, but at a slower rate than immature neurons and that change in sensitivity is Apaf1-dependent (Wright et al., 2007; Vaughn and Deshmukh, 2008). Moreover, endogenous expression of Apaf1 in mature

neurons is re-established following DNA damage, and dependent upon the availability of chromatin to the transcriptional machinery (Wright et al., 2007; Vaughn and Deshmukh, 2008). It is possible that the increased threshold to apoptosis protected the somas of the etoposide-treated adult cortical neurons, and that the toxicity was targeted to the neurites, resulting in the degeneration of the neural processes. Indeed, such notions have been described earlier following transient ischemia (Fukuda et al., 1999), endogenous toxicity in a an amyothrophic lateral sclerosis model (Gould et al., 2006) and a DNA damage repair mutant (de Waard et al., 2010). If true, it is possible that such effects may have been missed upon morphological evaluation. On the other hand, cleaved caspase-3 staining markedly identified the cytoplasm of cells undergoing apoptosis (Figs. 6, 7). It is also conceivable that ultrastructural changes were subtle at the 6 h time point for adult neuron apoptosis. Similarly, only 6 h may have been insufficient time to see mature neuron death. Indeed, 25-day cultured neurons acquired fewer DSBs, as determined by comet assay, compared to 5-day cultured neurons after 4 h of etoposide treatment (Martin et al., 2009). Further, the susceptibility to DNA damage-mediated apoptosis in mature neurons is slowed compared to immature neurons, thereby supporting such a notion (Wright et al., 2007).

In Chapter III, I used the pharmacological agent KU55933 (K5) as an inhibitor of ATM in order to establish ATM's role in ribosomal biogenesis. The selectivity and functionality of this inhibitor to ATM has been well-established as a competitive ATM binder (Hickson et al., 2004; Bryant and Helleday, 2006; Li and Yang, 2010). The reliance we have placed on K5 as an inhibitor of ATM is potentially problematic, but was

necessary because multiple attempts to knock down ATM using short hairpin RNA (shRNA) were unsuccessful. K5 activity in our cellular system has been identified by the inhibition of ATM autophosphorylation at S1981 in response to etoposide treatment, as indicated by Western blot analysis. The autophosphorylation site S1981 is not phosphorylated in response to non-DNA damage-related stimuli such as Gabazine or BDNF (data not shown). Therefore, ATM inhibition by K5 in response to these stimulations was not identifiable by Western blot, nor was K5 inhibition of ATM under basal conditions. Further, despite published K5 specificity to inhibit ATM in cycling cells, its use in neurons has not been previously reported. As such, K5's specificity to ATM in our neuronal system has only been assumed. It is therefore possible that K5 may be metabolized in our cellular system in a manner that reduces its specificity to ATM, thus suggesting that the K5 effects on nucleolar transcription could be partly mediated through signaling pathways that engage relatives of ATM, such as PI3K or mTOR. Both the PI3K and mTor are known to respond to neurotrophins and neuronal activity. However, in etoposide-treated neurons, I observed a K5-sensitive induction of rDNA transcription in the absence of any evidence for PI3K/mTor activation. Furthermore, the inhibition of mTOR by K006 for 24 h did not have the same effect on neurite outgrowth as did K5 treatment for 24 h. Most importantly, Pol1-driven transcriptional impairment was identified in the cerebellum of ATM-null mutant mice. This finding provided genetic loss of function data that paralleled Pol1-mediated transcriptional responses in K5-treated neuronal cultures and ICV infusion of K5 in the rat neonate neocortex. Therefore, it is unlikely that K5 treatment provided major inhibitory effects on Pol1 transcription by a means other than inhibiting ATM in our systems.

Our work has provided evidence that supports the involvement of ATM in nucleolar transcription as a far-reaching biological phenomenon. First, the recapitulation of nucleolar transcription suppression following K5-mediated ATM inhibition in vivo provides evidence that Pol1-mediated transcription in multiple cell types is incomplete without ATM involvement. As glia make up a significant portion of the neonate brain, this result suggests that ATM's involvement in nucleolar transcription may not limited to neurons. Broad biological involvement of ATM in nucleolar transcription is further evidenced by the work conducted in the HeLa and SHSY-5Y immortalized human cell lines. The potentiation of Pol1-mediated transcription in response to serum replenishment after serum starvation is attenuated by K5-mediated inhibition of ATM. The absence of ATM does not cause a complete inhibition of nucleolar transcription, but rather a reduction compared to basal levels, presumably via redundancy by other pathways that regulate Pol1. ATM deficiency in the cerebellum of 5-week-old ATM-null mice shows evidence of Pol1-mediated transcriptional suppression, while 11-week-old mutant mice show enhanced Pol1-mediated transcriptional inhibition (data not shown). At 11-weeks of age the cerebellar Purkinje cells have started to degenerate in the ATM-null mutant mouse lines (Li et al., 2011). Neurodegeneration is known to stimulate reactive gliosis (Smith et al., 2011). As such, we hypothesized that the increased 45S rRNA levels were due to reactive glia responding to the neurodegeneration, and therefore evaluated the levels of glial fibrillary acidic protein (GFAP) at both the 5-week and 11-week ages. These results showed that GFAP levels were no different in 5-week ATM-null mice compared to wildtype, but were significantly increased in the 11-week old mice compared to wildtype (data not shown). This may suggest that in the absence of ATM,

reactive glia were able to increase nucleolar transcriptional presumably using other pathways such as mTor or ERK1/2. Importantly, it does not suggest that the ATM-mediated suppression of Pol1 is rescued by reactive gliosis, but rather that the increased growth of dividing cells masks the net effect on Pol1.

Activation of ATM is identified by its autophosphorylation at S1981, the canonical DNA damage-mediated activation site (Bakkenist and Kastan, 2004). Attempts to show ATM autophosphorylation at S1981 following BDNF or synaptic activity stimulation have been unsuccessful. Though critics may interpret such a finding as evidence for ATM unresponsiveness to trophic stimuli, an alternative interpretation that considers other supporting data is that non-DNA damage-related stimuli activate ATM without affecting autoactivity at S1981. Indeed, ATM has been shown to have multiple autophosphorylation sites (Kozlov et al., 2006; Kozlov et al., 2011). Moreover, evidence of ATM activation in response to oxidative stress has been shown to be separate from its activation in response to double-stranded breaks. Indeed, the ATM dimer is activated by redox reaction in response to oxidative stress instead of by phosphorylation of an ATM monomer (Guo et al., 2010a; Guo et al., 2010b).

Due to the involvement of nucleolar transcription in neurite outgrowth (Gomes et al., 2011), we assessed the effect of 24 h pharmacological inhibition of ATM on neurite outgrowth in developing neurons in culture compared to vehicle and mTOR inhibition treatments. Both mTOR and ATM inhibition suppressed neurite outgrowth under basal conditions. ATM inhibition under BDNF-stimulated conditions also robustly suppressed neurite outgrowth. These results suggest a downstream biological effect on neurites following sustained nucleolar transcriptional suppression, and support the notion that

ATM is a growth-promoting kinase (Yang and Kastan, 2000; Li and Yang, 2010). However, as ATM has been shown to be involved in insulin signaling and translation initiation (Yang and Kastan, 2000; Li and Yang, 2010), and mTOR is a well-established upstream mediator of protein synthesis and cellular growth (Mayer et al., 2004; Ruvinsky and Meyuhas, 2006; Swiech et al., 2008), the ant-growth effects of K5 and K006 may also involve these additional effectors besides Pol1. For instance, 24 h pharmacological inhibition of ATM and/or mTor may have reduced phosphorylation of 4E-BP1, and thus decrease translation (Mayer et al., 2004; Ruvinsky and Meyuhas, 2006). Further, it is possible that the effects we have witnessed in neurite morphogenesis are the outcome of changes in cytoskeletal dynamics in response to ATM inhibition mediated through an interaction with nuclear factor kappa B (NF-KB) (Gutierrez and Davies, 2011; Semlitsch et al., 2011).

Result artifacts can stem from the system with which we've been working, despite having controls in the same system. Harvesting primary cortical neurons requires trituration to separate cell clumps. This process is an unnatural one, and thus may cause cellular damage. Further, in order to grow an enriched population of cortical neurons, glia proliferation must be suppressed. Arabinofuranosyl cytidine (AraC), a genotoxin, is used to prevent the growth of dividing glia cells in our culture system. Though AraC has been widely used, including in the Hetman lab, and it does not appear to have negative effects on neurons at the concentrations used, it may theoretically activate DNA damage response contributing to the apparent ATM signaling in response to non-genotoxic stimuli. Perhaps the greatest source of artifact, because of ATM's ability to respond to oxidative stress, is the non-physiological oxygen/CO_2 conditions of the incubators in

which the neuronal cultures were maintained. The 95% air/5% CO_2 content may generate oxygen radicals that activate ATM in a manner that will not induce its autophosphorylation at S1981, but nonetheless induce ATM activity (Guo et al., 2010a; Guo et al., 2010b). These complications imply further importance on the recapitulation of the key *in vitro* results in the whole animal. Supporting these *in vitro* results, we observed suppressed Pol1-mediated transcriptional activity in the $Atm^{-/-}$ cerebella, and in whole rat neonates after infusion of K5 intracerebroventricularly.

Etoposide as a novel suppressor of neuronal Pol1

Etoposide inhibits Topo2 causing single-stranded breaks in DNA and DNA-Topo2 adducts, as well as double-stranded breaks (Bromberg et al., 2003; Pietrzak et al., 2011b). Oxidative- or energy deprived-mediated cellular stress-derived impairment of nucleolar transcription can be mediated by JNK2 or AMPK, respectively (Mayer et al., 2005; Hoppe et al., 2009). Genotoxic stress-induced Pol1 transcriptional inhibition is further dependent upon the presence of ATM (Kruhlak et al., 2007), and may also be a response to physical interference caused by DNA lesions (Hetman et al., 2010). Regardless of the mechanistic detail, the transcriptional pause offers an opportunity to conserve energy, prevent cell cycle entry and growth, and provides an opportunity to respond and perhaps repair genotoxic damage.

In cycling cells, nucleolar transcription halts and the nucleolus disappears upon mitotic entry by the phosphorylation of TAF_I110 at T852 by Cdk1-CyclinB (Drygin et al., 2010; Grummt, 2010). This phosphorylation impairs the SL1/TIF-1B interaction with UBF to prevent initiation complex formation. This SL1/TIF-1B – UBF interaction is also prevented by JNK2 phosphorylation of TIF-1A at T200 in response to oxidative stress

(Mayer et al., 2005). JNK2 signaling to impair nucleolar transcription may be specific to oxidative or bulky adduct lesions as irradiated *JNK2* deficient cycling cells continue to transcribe rDNA (Kruhlak et al., 2007). Though DNA damage may cause physical impairments on DNA that prevents promoter binding (Marietta et al., 2002) or transcriptional elongation (Hetman et al., 2010), irradiation-mediated DNA damage may cause Pol1 transcriptional inhibition by the activity of DNA damage repair enzymes such as ATM (Kruhlak et al., 2007). While my data does not support the role of ATM as a suppressor of Pol1, a possibility that JNK2 or CDKs play such a role in etoposide-exposed nervous system is worth further testing.

Etoposide treatment required high concentrations to suppress Pol1-mediated transcription in cortical neurons cultured from newborn rats, and in neonate and adult brains. Though we concentrated our analysis on postmitotic neurons, *in vivo* immunohistochemical staining of NPM/B23 suggested that all analyzed cellular nucleoli were disrupted. This would suggest that at the etoposide concentrations used, the nucleoli of glia were equally susceptible to nucleolar disruption as were neurons. In contrast, researchers using wildtype MEFs treated with 20 μM etoposide showed Pol1-mediated transcriptional inhibition (Kruhlak et al., 2007), while $Atm^{-/-}$ MEFs did not. These results suggest that the response to etoposide is akin to the irradiation response, even at low concentrations of etoposide (Kruhlak et al., 2007). Though this may initially appear like a discrepancy, it would suggest that dividing cells are more susceptible to genotoxic damage than postmitotic cells, such as neurons. The etoposide concentrations used in the neonate and adult brain exceeded the threshold for glial transcriptional inhibition (perhaps around 20 μM, or in the brain 2 nmoles), whereas postmitotic cells

required higher concentrations of the single-stranded breaks and bulky adducts for nucleolar disruption. Therefore, it may be no surprise that our observations could not identify non-neuronal cells that had completely intact nucleoli. Further, in the neonate, 10 nmoles of etoposide resulted in an incomplete disruption, as evidenced by diffusion of NPM/B23 in the nucleoplasm with some remaining punctuate staining foci of NPM/B23 in the nucleolus.

ATM as a novel positive regulator of Pol1

It may not be surprising that ATM can influence the activity of Pol1 given its downstream influence over the cell cycle in response to double-stranded DNA breaks. Indeed, Pol1-mediated transcription is ongoing in MEFs lacking ATM, and in *ATM*$^{-/-}$ cells in the presence of irradiation or etoposide treatment, while other DNA damage signaling kinases are dispensable (Kruhlak et al., 2007). These data suggest that ATM transiently inhibits the assembly of the Pol1 initiation complex following DNA damage by a yet unknown mechanism, and leads to displacement of elongation enzymes. Within one hour, MEFs exposed to irradiation or etoposide recovered from Pol1-mediated transcriptional arrest. The activation of ATM is likely mediated by the DNA torsional strain applied by the induction of bulky adducts by irradiation or etoposide, as ATM is autophosphorylated by chromatin rearrangement (Bakkenist and Kastan, 2003; Berkovich et al., 2007; Iijima et al., 2008). One may hypothesize that this response is mediated by JNK2, as the nucleolar stress signaling kinase (Mayer et al., 2005; Mayer and Grummt, 2005), but in these conditions the nucleolar stress response requires ATM while JNK2 is dispensable (Kruhlak et al., 2007). The DNA damage-mediated suppression of Pol1 and subsequent nucleolar disruption, which ultimately leads to p53 accumulation, may be an

important step to promote a p53-ATM interaction with an active ATM to potentiate a stress response signal transduction cascade.

Data presented herein support the hypothesis that ATM is a regulator of Pol1-mediated transcription, but in a manner outside the realm of DNA damage signaling. Kruhlak, et al. suggest that ATM activation impairs Pol1 transcriptional activity and ATM deficiency prevents Pol1 transcriptional suppression. Conversely, data presented here suggest that ATM is a regular contributor to induce Pol1-mediated transcription and its absence suppresses Pol1-mediated transcription when compared to basal levels. Though this finding may appear to be a contradiction to the Kruhlak, et al. data, upon further evaluation of that report, it is clear that basal levels of Pol1 transcription were not quantified and compared between ATM deficient and control cells. Therefore, it is possible that in the ATM deficient MEFs and fibroblasts, Pol1-mediated transcription was basally impaired compared to control cells. Continuing in that logic, Pol1-mediated transcription would have remained viable due to canonical Pol1 regulators, such as ERK1/2, PI3K and mTOR. Etoposide likely activated ATM in response to Topo2 pause that causes DNA torsional strain (Bakkenist and Kastan, 2003). The low etoposide dose (1 μM) and short kinetics (4 h) likely contributed to my ability to detect the induction of Pol1-mediated transcription. It is possible that ATM was immediately and locally activated in response to the DNA torsional strain, which may have loosened chromatin at the site of damage either by supercoiling or by recognition of the damage and recruitment of responding enzymes, such as the MRN complex. Despite the complexities associated with ATM activation following DNA damage, etoposide may cause dichotomous ATM behavior depending upon its kinetics and concentration.

Despite the majority of our work being conducted in cultured, primary neurons obtained from newborn rats, we extended our findings to the neocortex of rat neonates, as well as cycling, immortalized human cell lines. Indeed, the basal suppression of Pol1-mediated transcription in response to ATM inhibition is robust enough to be identified in RNA extracted from whole brain lysates, of which multiple cell types are present. Further, these findings were recapitulated in ATM deficient mouse cerebella, the RNA of which was also extracted from whole cerebellar lysates. The findings herein suggest that the ATM-mediated contribution to Pol1-mediated transcription is ubiquitous. This suggestion may even be extrapolated to previous reports of ATM being required for etoposide-mediated nucleolar suppression in MEFs (Kruhlak et al., 2007). Had these researchers been evaluating nucleolar transcription following a detailed etoposide dose response, they may have also witnessed the finding we report herein. However, the Pol1-mediated suppression response to ATM deficiency can be masked by other signaling pathways that contribute to Pol1-mediated transcriptional activity. An example of this masking can be taken from the glial response to neurodegeneration in ATM-deficient mice. Growth is required by glial cells to fill in the tissue gaps created by atrophying neurites. The stimulation of Pol1-mediated transcription by PI3K/mTOR and/or ERK-MAPK is sufficient to stimulate growth, but would be less stimulatory to Pol1 transcription than an equally reactive gliosis in ATM wildtype brain that also had PI3K/mTOR and/or ERK-MAPK input. The ATM input, therefore, may also be growth-related, and thus have an upstream activator sufficient to provide such growth-stimulated input.

Great progress has been accomplished in recent years in identifying the DNA damage-mediated activation of ATM. Relaxation of chromatin appears to be the activating stimulus of ATM, which is a homodimer prior to autophosphorylation at S1981 (Bakkenist and Kastan, 2003). Despite the presence of numerous DNA damage-initiated autophosphorylations (Kozlov et al., 2011), once autophosphorylated at S1981 the dimerized ATM dissociates and its monomers are recruited to the sites of damage. The dimerized form of ATM is integral for its capacity to signal in response to oxidative stress (Guo et al., 2010b). ATM oxidation causes formation of a disulfide bond cross-linker at a specific cysteine (C2991) between the two monomers, yet the responsiveness of ATM to DNA damage is unaffected by site-directed mutagenesis of C2991 (Guo et al., 2010b). We have observed that S1981 autophosphorylation is absent in synaptic activity-, or BDNF-stimulated neurons (data not shown), yet ATM inhibition suppresses the Pol1-mediated transcriptional drive associated with these processes. This suggests that ATM could be a dimer in regulating nucleolar transcription. Further support for this possibility comes from the basal levels of nucleolar transcription that are impaired upon ATM inhibition. What then could be the possible upstream activator of the dimer form of ATM? Upstream activation of PI3K-like family members such as PI3K and mTOR can be mediated by small GTPases (Long et al., 2005; Aspuria et al., 2007). The amino acid-mediated activation of mTOR is thought to be mediated by the small GTPase, Rheb (Inoki et al., 2005; Long et al., 2005). Rheb is ubiquitously expressed in all tissues, but particularly enriched in the brain (Inoki et al., 2005). Indeed, it is the brain enrichment that is its namesake: Ras homolog enriched in brain. The Rheb interaction with mTOR has linked it to S6K and 4EBP1 activation (Inoki et al., 2005). Interestingly, this GTPase

has been further shown to interact with ATM (Inoki et al., 2005). Though the function of this interaction is not known, such a discovery offers an attractive activation mechanism possibility for ATM-regulated nucleolar transcription. As Rheb has already been identified in activating mTOR in response to nutrient presence and inducing S6K and 4EBP1 activation (Inoki et al., 2005; Long et al., 2005), it is conceivable that Rheb could activate ATM to stimulate nucleolar transcription in response to cellular need. A potential problem with this hypothesis is that Rheb is cytoplasmic – activated by the GTPase activating protein (GAP), TSC2, whereas ATM is enriched in the nucleolus, near its transcriptional target. Though conceivable that Rheb could shuttle to the nucleolus, another GTPase, Nog1, resides in the nucleolus and regulates rRNA maturation (Park et al., 2001; Lapik et al., 2007). Though there is no evidence of interaction with ATM, Nog1 complex formation has been shown to be regulated by mTOR and function in the nucleolus to mediate late ribosomal maturation (Honma et al., 2006). These findings provide enough circumstantial evidence to warrant further investigation of Nog1 or Rheb as an upstream activator of ATM-mediated nucleolar transcription.

The nucleolar ATM target to induce Pol1-mediated transcription is currently unknown. A search of phosphorylation sites on nucleolar transcriptional machinery offers possibilities that include NPM/B23 (Li et al., 2007; Nalabothula et al., 2010), and a proteomics report of an undescribed interaction between SIRT7 and ATM (Tsai et al., 2011), while others have reported irradiation-mediated phosphorylation of TAF1C, and TTF1 by ATM (Matsuoka et al., 2007). TAF1C is a component of SL1/TIF-1B, involved in regulating the interaction between Pol1 and the rDNA promoter and required for transcriptional elongation (Anney et al., 2010; Drygin et al., 2010). The other potential

ATM targets (TTF1, NPM/B23 and SIRT7) may be intimately related to the regulation of Pol1-mediated by ATM. NPM/B23 is a particularly attractive choice as its histone chaperone activity has been identified to enhance Pol1-mediated transcription by binding to the chromatin surrounding the rDNA promoter (Murano et al., 2008). Further, the NPM/B23 S125 residue has been identified as an ATM/ATR/SMG phosphorylation site (Maiguel et al., 2004; Nalabothula et al., 2010). In addition, the abundance of NPM/B23 is directly proportional to the rate of transcription, perhaps to maintain the euchromatin structure (Murano et al., 2008). Evidence suggests that NPM/B23 is upregulated in ATM deficient cells (Nalabothula et al., 2010), the reasons for which are not yet entirely clear (Colombo et al., 2011). A simple explanation for this observation could be that because p53 is increased in ATM-deficient cells (Kamijo et al., 1999), the increased expression of NPM/B23 is a response to negatively regulate p53 stabilization (Nalabothula et al., 2010).

Additional ATM targets include TTF1 and SIRT7. TTF1 is one target potentially intimately related to NPM/B23. NPM/B23 is used as a chaperone to shuttle a nucleolar localization sequence-bound TTF1 from the nucleoplasm to the nucleolus to initiate rDNA transcription. In contrast, ARF prevents TTF1 from accumulating in the nucleolus by blocking the nucleolar localization sequence, thereby preventing rDNA transcription (Lessard et al., 2010). A recent proteomics study identified an interaction between ATM and SIRT7 (Tsai et al., 2011). This interaction was presented in a supplemental data list of proteins that showed positive interaction with SIRT7. However, SIRT7 has been shown to interact with UBF and is necessary for sufficient Pol1 transcriptional activity (Ford et al., 2006; Grob et al., 2009) by its promotion of chromatin remodeling (Tsai et

al., 2011). Perhaps the chromatin remodeling function of SIRT7 activates ATM, which subsequently phosphorylates NPM either to initiate TTF1 nucleolar localization, or as part of a previously unidentified initiation complex.

Whatever the upstream activator and nucleolar target is, the nucleolar enrichment of ATM may be functionally important. Among its other functions, ATM can be included as a growth-stimulating kinase (Yang and Kastan, 2000; Li and Yang, 2010). Adding ATM to the other pathways that positively regulate rDNA transcription provides further layer of regulation of an extremely important process. What about ATM favored its contribution to this redundant activity? During evolution, did the primary functions of ATM include Pol1 regulation as may be indicated by its nucleolar enrichment? Can ATM as SMG-1 (also known as ATX) respond to damage and or secondary structures in RNA and if so, could rRNA processing activate an ATM-dependent auto feedback loop that drives Pol1? These are all intriguing questions that if answered may provide additional information about this novel positive regulator of nucleolar transcription.

Consequences of nucleolar dysfunction

Dysfunctional ribosomal biogenesis has an obvious consequence: ribosomal renewal will be halted or insufficient. If ribosomal renewal is necessary for the sufficient translation of genes, then its impairment will negatively impact cellular maintenance (Bramham and Wells, 2007; Besse and Ephrussi, 2008). Growth will be impaired (Grummt, 2010), as will be the re-initiation of the cell cycle (Drygin et al., 2010). In postmitotic neurons, dendritic arborization and outgrowth, as well as axon guidance and local protein synthesis will be negatively impacted with dysfunctional ribosome synthesis (Alvarez et al., 2000; Cox et al., 2008; Gomes et al., 2011), resulting in the impaired

communication between the nucleus and distant synapses. Further, protein synthesis-dependent forms of synaptic plasticity such as LTP would be impaired (Ostroff et al., 2002; Malenka, 2003).

Yet implied in the singular impairment of ribosomal biogenesis are other consequences not related to protein synthesis. Impaired rDNA transcription disrupts nucleolar integrity allowing nucleolar proteins to diffuse into the nucleoplasm and allow the accumulation of p53, which can cause apoptosis (Olson, 2004; Boulon et al., 2010). Further, the reorganization of the nucleolus following mitotic nucleolar disruption is brought on by CDC14B, a phosphatase that reactivates SL1/TIF-1B after mitosis in cycling cells (Drygin et al., 2010). Without the cooperation of a Cdk4-cyclin D/Cdk2-cyclin E/A-mediated active UBF in early G1-phase, however, Pol1-mediated transcription, would not be optimized. The post-mitotic reorganization process, like stress-induced nucleolar disruption, requires multiple steps that could be dysregulated if cellular stress caused the disruption (Angelier et al., 2005; Boulon et al., 2010; Hernandez-Verdun, 2011). Disassembly of the nucleolus in prophase includes the phosphorylation of NPM/B23 by Cdk1-cyclin B to reduce its RNA-binding affinity. During anaphase RNA binding affinity is restored by the phosphatase, PP1 (Negi and Olson, 2006; Hernandez-Verdun, 2011). Interestingly, in ATM deficient cells, PP1 dephosphorylation of NPM/B23 is dysregulated, thus allowing for the overexpression of NPM/B23, as well as its increased expression, and increased phosphorylation of S125, leading to reduced p53 degradation (Tang et al., 2008; Nalabothula et al., 2010). This same phosphorylation site is an interphase casein kinase 2 (CK2) phosphorylation site that regulates the interaction of NPM/B23 with other nucleolar proteins during nucleolar

re-assembly thereby contributing to the rate at which the nucleolus assembles during telophase (Negi and Olson, 2006). This may suggest that in ATM deficient cells where p53 accumulation is increased (Kamijo et al., 1999) there is also a reduced reassembly of the nucleolus in telophase compared to wildtype cells, as the phosphorylation-mediated assembly could be mediated by changes in dissociation constants. In addition, if a stressful stimuli induces NPM/B23 translocation from the nucleoplasm, this may suggest that NPM/B23 or its upstream activator, Cdk1, is a substrate of stress-induced JNK2, which regulates the nucleolar stress response. The stress-induced phosphorylation by JNK2 on TIF-1A at T200 impairs the interaction between SL1/TIF-1B and Pol1 thereby preventing the initiation complex from forming on the rDNA promoter. Indeed, NPM/B23 translocation is also dependent upon JNK2 in response to UV exposure (Yogev et al., 2008). These findings suggest that the phosphorylation status of NPM/B23 may regulate the rate at which the nucleolus can be assembled and disassembled, thus mutations of NPM/B23 could have profound effects on the function and presence of the nucleolus. A recent discovery has identified the intergenic spacer regions of rDNA as a generator of stress-specific, inducible, large non-coding RNAs (lncRNA) that regulate the passive diffusion of specific proteins between the nucleolus and nucleoplasm. The lncRNAs capture proteins that display a peptidic code termed the nucleolar detection sequence (NoDS) and keep them in the nucleolus (Audas et al., 2012). This new mechanism adds yet another layer of complexity to the nucleolar stress response and begins to mechanistically explain the specific diffusion of proteins under specific stresses.

In postmitotic neurons, genotoxic stress by topoisomerase I or II poisons impairs nucleolar transcription and causes p53-mediated apoptosis in neonates. If the exposure to a nucleolar-sensitive genotoxic stressor is sublethal and transient, there is evidence to suggest that the nucleolar response is transient also (Zhang et al., 1988; Casafont et al., 2011). Indeed, within 1 day of IR-induced DNA damage to sensory ganglion neurons, nucleolar transcriptional activity resumes (Casafont et al., 2011). This suggests that the neuronal nucleolar transcription suppression we observed in etoposide-treated rats would likely have resumed had it been evaluated 1 day post treatment. Indeed, at least in rat neonates, we observed the resumption of an intact nucleolus as identified by the nucleolar presence of B23 immunostaining at 72 h post ICV etoposide delivery. Therefore, transient inhibition of nucleolar transcription may not induce long-term neuronal damage in the mature nervous system. Support for this notion comes from human neurotoxicity studies of chemotherapy agents, such as etoposide. For instance, the neurological side effects are relatively infrequent and short lasting following etoposide delivery through the intracarotid artery to treat brain tumors (Madajewicz et al., 2000). The few ill effects noted include headache, focal seizure, blurred vision, and, most commonly, transient confusion. This latter effect was reported in only 8 of 168 treated individuals (Tfayli et al., 1999; Madajewicz et al., 2000). In addition, a long-term animal study evaluated cognitive impairment following 18-week long regiments of chemotherapy delivered by five systemic injections every four weeks (Lee et al., 2006). One of the chosen drugs, 5-FU, is known to cause nucleolar stress (Rubbi and Milner, 2003). Surprisingly, after a 7-week recovery, LTP was enhanced, but subsequently returned to control levels at 42 weeks. It is tempting to speculate that the initial enhancement of LTP was due to highly

active ribosomal biogenesis during the rebound phase following its inhibition during chemotherapy. This increased rate of ribosomal renewal may have replaced numerous damaged ribosomes with impaired function. With increased numbers of functioning ribosomes, local protein synthesis may have been enhanced and successful communication between the nucleus and synapses enhanced. Further, increased accumulation of p53 in the nucleus following nucleolar disruption is likely to have ceased, and along with it the ceasing of enhanced stress-mediated responses. Therefore, if in the mature nervous system the impairment of Pol1-mediated transcription is transient, it may produce no residual neuronal deficits (Fig. 17). To be certain, more work is required to quantify the cellular recovery rate by genotoxin type, delivery and dose, as well as by recovery period and age at treatment. Indeed, reports of chemobrain, also termed chemotherapy-induced cognitive impairment, are most commonly associated with treatments of breast cancer, but remain elusive as to the triggers, treatment conditions that potentiate it, and duration (Kannarkat et al., 2007; Whitney et al., 2008; Argyriou et al., 2010; Mitchell and Turton, 2011). Though the frequency of associations are ever increasing, little is understood about the susceptibility of patients to chemobrain. This is partially due to the primarily descriptive and associative nature of chemobrain reports, but also confounded by the variation in ages of chemotherapy onset and post treatment longevity (Argyriou et al., 2010).

The effects of genotoxic stress on postmitotic neurons could be compounded by age as they are postmitotic and dependent on translation for cellular maintenance and synaptic plasticity. Therefore, it may be anticipated that progressive, impaired ribosomal biogenesis could result in dysfunction or neurodegeneration. Indeed, the repetitive nature

of the rDNA tandem repeats increases the susceptibility of rDNA to recombination, and has been associated with regulating senescence in yeast (Kobayashi, 2011). Moreover, mammalian age-related rDNA gene loss were described in the early 1970s (Johnson and Strehler, 1972), suggesting that impaired ribosomal biogenesis is a consequence of natural aging. Further, age-related loss of rDNA has been observed in the human cerebral cortex (Strehler et al., 1979), and 5S and 28S rRNA genes have been shown to be preferentially lost in human adipose tissue during natural aging (Zafiropoulos et al., 2005). Interestingly, rDNA loci were increased in the parietal and prefrontal cortex of postmortem Alzheimer's Disease patients, compared to age-matched controls, as was cytosine methylation at the rDNA promoter (Pietrzak et al., 2011a). This could be explained by increased stability of silenced, hypermethylated rDNA. Indeed, increased cytosine methylation has been shown to stabilize yeast rRNA genes (Kennedy et al., 1997). Further, prematurely senescent primary fibroblasts from human Werner syndrome (WS), a progeroid disorder, did not display any locus instability, but showed increased cytosine methylation within rRNA genes (Machwe et al., 2000).

The DNA damage repair pathway, nucleotide excision repair (NER), has recently been associated with rDNA transcription (Assfalg et al., 2011). Mutations of TFIIH subunits associated with Cockayne syndrome impair rDNA transcription. The authors indicate that TFIIH, a general transcription factor of RNA Polymerase II, is essential for rDNA promoter occupancy and Pol1-mediated transcriptional elongation. Indeed, these findings suggest that the progeroid disorder, Cockayne syndrome, and perhaps other mutations of NER that cause progeria and cancer predisposition have associations with impaired ribosomal biogenesis.

Recent years have brought growing evidence for an association between reduced ribosomal biogenesis and neurodegenerative diseases. Tamoxifen-induced ablation of Tif-1a in the mature CNS potentiates noticeable cell loss in the dentate gyrus and CA regions of the hippocampus beginning at one month post tamoxifen treatment (Parlato et al., 2008). The ablation of Tif-1a in mature mouse dopaminergic neurons leads to reduced cell number and a Parkinsonian phenotype that can be rescued by L-Dopa administration (Rieker et al., 2011). Indeed, translocation of NPM/B23 from the nucleolus to the nucleoplasm and decreased visibility of nucleoli has been observed in dopaminergic neurons of postmortem Parkison's patients (Rieker et al., 2011). In the CAG expanded repeat disorder, Huntington's Disease (HD), dysfunctional UBF acetylation impairs UBF occupancy on the rDNA promoter in a mouse model of HD. In addition, these authors observed reduced UBF protein levels in human postmortem striatum (Lee et al., 2011). In Alzheimer's Disease (AD), the presence of decreased polyribosomal function has been described in the postmortem parietal cortex (Ding et al., 2005). The ribosomal dysfunction is at least partially associated with increased rRNA oxidation (Ding et al., 2006). Further, increased rRNA oxidation by iron binding has been identified in the AD postmortem hippocampal cytoplasm. Moreover, AD associated oxidative damage resulted in 8-hydroxyguanosine insults, which were not witnessed in age-matched controls (Honda et al., 2005). There has also been neuronal nucleolar atrophy, a symptom of reduced Pol1-mediated transcriptional activity, identified in AD postmortem hippocampi and cerebral cortices (Tagliavini and Pilleri, 1983; Mann et al., 1988; Iacono et al., 2008). In addition, Prader-Willi Syndrome (PWS) patients and mouse models of PWS have recently been shown to have atrophic Purkinje cell nucleoli

(Leung et al., 2009). Interestingly, published electron micrographs may suggest the absence of nucleoli in Purkinje cells of Atm-/- mice, indicating that the cerebellar neurodegeneration associated with ATM deficiency may be associated with nucleolar disruption (Kuljis et al., 1997). Further, deficiency of ATM results in increased accumulation of iron and subsequent increased accumulation of oxidative stress (McDonald et al., 2011). Thus, the absence of ATM may potentiate the rRNA-targeted oxidative stress and ribosomal dysfunction. These findings are particularly interesting in light of results presented herein pertaining to the regulation of Pol1 transcription by ATM, as it suggests that long-term (i.e., life-time of the animal) insufficient ribosomal biogenesis may cause a permanent collapse of nucleoli leading to cellular degeneration. Therefore, the renewal of neuronal ribosomes may be necessary to prevent consequences of oxidative stress including ribosomal failure. Conversely, chronic nucleolar transcriptional impairment may turn rRNA to be an oxidative stress sink. This notion favors a long accumulation of oxidative insult with pathology evidenced after years, consistent with many neurodegenerative diseases, including A-T. Importantly, these nucleolar associations with neurodegenerative disease do not claim to be causative. Though deletion of Pol1 transcription (Rieker et al., 2011) is able to cause the symptoms associated with PD as a proof of principle, the ablation of nucleolar transcription is unlikely. Therefore, additional work is required to identify biological consequences of nucleolar insufficiency in neurodegenerative conditions such as A-T.

The nucleolus as a therapeutic target

The breadth of functions associated with the nucleolus make it an enticing candidate for targeted therapies. NPM/B23 is an exciting potential therapeutic target because it has the capability of promoting nucleolar disruption, which could be useful in cancer therapy, while it also has the potential to promote nucleolar assembly and euchromatin formation. The phosphorylation of NPM/B23 can cause disruption of the nucleolus, which, if targeted and constitutive, would cease tumor growth. Conversely, constitutive phosphorylation at S125 may promote rapid reassembly of a disrupted nucleolus that could prevent transient or permanent cellular damage. Similarly, as the phosphorylation of these residues are mediated by Cdks and CK2, respectively, these kinases may offer further target potential to manipulate the structural status of the nucleolus through NPM/B23. NPM/B23 has already been shown to be a downstream target of ATM (Nalabothula et al., 2010), presumably under conditions of cellular stress, but it may also be a target that mediates Pol1-mediated transcriptional regulation by ATM. Constitutively activating NPM/B23 at S125 may rescue insufficient nucleolar transcription in ATM deficient cells. Indeed the number of protein-protein interactions with ATM in the nucleolus is regularly increasing. SIRT7 is one such newly discovered ATM-interacting protein (Tsai et al., 2011) that has been shown to be essential for nucleolar transcription (Ford et al., 2006; Grob et al., 2009). Thus, SIRT7 may be another target of ATM to promote nucleolar transcription. Though SIRT7 has been shown to have very limited deacetylase activity (Tsai et al., 2011), it is required for Pol1 transcription (Grob et al., 2009). Given the attention paid to sirtuins in the pharmaceutical industry, this target may be a low-hanging fruit.

Components of the Pol1 initiation complex are convenient targets for potential therapies as well, with potentially numerous substrates that affect transcriptional initiation and elongation. Indeed, constitutively active TIF-1A has been effective way to selectively stimulate Pol1 under numerous conditions (Grummt, 2010; Gomes et al., 2011). Many groups have already attempted to target the phosphatase CDC14B for its dephosphorylation of SL1/TIF-1B to reset the Pol1 initiation complex after mitosis (Mocciaro and Schiebel, 2010). This phosphatase remains an interesting target, as does SL1/TIF-1B in the field of cancer, as tumorigenesis would presumably not resume if CDC14B were unable to reactivate SL1/TIF-1B. However, overexpression of CDC14B is now thought to be oncogenic, suggesting multiple functionality of this phosphatase (Wei and Zhang, 2011). UBF has been identified in HD patients as being poorly expressed, thus therapies that may increase its expression and interactions with the rDNA promoter and Pol1initiation complex may prove beneficial.

Given the recent associations between nucleolar dysfunction and disease there is hope that therapeutic interventions targeting the nucleolus will emerge. Indeed the plurifunctionality of the nucleolus is becoming more and more evident. Therefore, I speculate that the list of genes that regulate nucleolar function and are associated with diseases will only become longer, thus prompting more development efforts towards therapies that target the nucleolus.

Figure 17

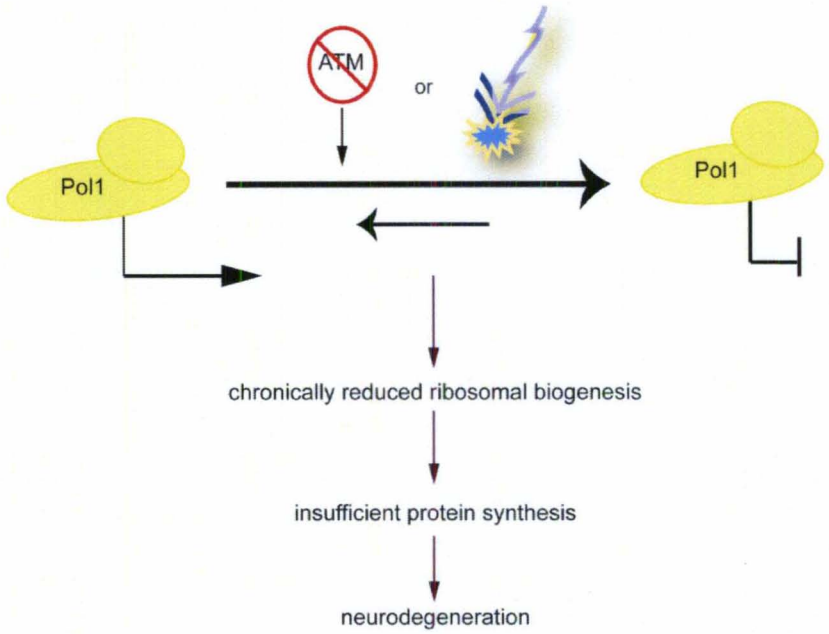

Figure 17. Chronic nucleolar transcriptional impairment leads to neurodegeneration
Sustained Pol1-mediated transcriptional inhibition by either persistent genotoxic insult or ATM deficiency leads to decreased sufficiency of protein synthesis. The persistence of these events may lead to neurodegeneration.

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PEER-REVIEWED PUBLICATIONS
Smith SC, Robinson AR, Niedernhofer LJ, Hetman M (2011). Downregulation of cholesterol biosynthesis genes in the forebrain of ERCC1-deficient mice. *Neurobiology of Disease*. http://dx.doi.org/10.1016/j.nbd.2011.12.036

Potts LF, Luzzio FA, **Smith SC**, Hetman M, Champy P, Litvan I (2011). Annonacin in *Asimina triloba* fruit: Implication for neurotoxicity. *Neurotoxicology. 33(1):53-58.*

Pietrzak M*, **Smith SC***,Geralds JT, Hagg T, Gomes C, Hetman M (2011). Nucleolar disruption and apoptosis are distinct neuronal responses to etoposide-induced DNA damage. *Journal of Neurochemistry.doi: 10.1111/j.1471-4159.2011.07279.x* *Equal contribution.

Gomes C, **Smith SC**, Youseff M, Zheng J-J, Hagg T, Hetman M (2010). RNA-Polymerase-1-driven transcription as a mediator of Brain-Derived Neurotrophic Factor (BDNF)-induced neurite outgrowth. *Journal of Biological Chemistry. doi:10.1074/jbc.M110.170134*

DISSERTATION CHAPTERS YET UNPUBLISHED
Regulation of RNA Polymerase-I-mediated transcription by ataxia teleangiectasia mutated (ATM)

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Emory University Winship Cancer Center (2011). "The moonlighting of DNA repair enzymes"

Cincinnati Children's Hospital (2011). "The moonlighting of DNA repair enzymes"
10th Annual Midwest DNA Repair Symposium, Univ. of Pittsburgh, Pittsburgh, PA (2008). "RNA microarray and histological analysis of ERCC1-deficient mouse brains indicates cerebellar neurodegeneration."
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OTHER ORAL PRESENTATIONS
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"Molecular insights into aging", Department of Neurosurgery, University of Louisville School of Medicine, 2009.
"Molecular insights into aging", Department of Neurosurgery, University of Louisville School of Medicine, 2008.

PATENTS
Smith SC United States Patent No. 6,955,093. Rupture testing for gloves. Issue date: October 18,2005.

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"ATM is a novel regulator of nucleolar transcription"

ASBMB Annual Meeting, Washington, D.C., USA
"ATM is a novel regulator of nucleolar transcription"

2010: 12th Annual Midwest DNA Repair Symposium, Louisville, KY, USA
"The sensitivity of nucleolar transcription to neuronal DNA damage"
The paper associated with this poster has been published in the Journal of Neurochemistry.

2009: The Society for Neuroscience Annual Meeting – Chicago, IL, USA
"Copper and zinc chelating protects neurons from DNA damage-induced transcription inhibition: Neuroprotective insights from a progeroid DNA damage repair mutant"
The paper associated with this poster is currently in preparation.

2008: 10th Annual Midwest DNA Repair Symposium - Pittsburgh, PA, USA
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Bridge customer needs to technical features
Identify competition and differentiating product features
Identify and direct opportunities for growth
Establish product pricing for business unit
Attend trade shows, design booths, establish end-user incentives for visiting the booths
Design and evaluate new products based upon end-user needs
Technical advising for Regulatory Affairs, Operations, and Quality Assurance

Accomplishments:
Brought to market and launched UltraSense™ and ComfortGrip™
Participated in directed growth of UltraSense™ to $1M during first 3 months in market
Directed forecasts of DiamondGrip™, DiamondGrip Plus™ and NitronOne™ brands to reflect product lifecycles
Established and ran focus group studies for MidKnight™ and new glove dispenser designs
Drove forecasting of product to reflect intermittent quality concerns

Translated archived product performance measures into meaningful marketing tools
Developed and implemented novel evaluation methods in analytical chemistry and material science to be used in marketing materials
Worked directly with Asian manufacturers to establish and validate evaluation procedures